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EXPERIMENTAL INTRADERMAL PNEUMOCOCCUS INFECTION IN RABBITS.

By KENNETH GOODNER.

(*From the Department of Bacteriology and Immunology of Harvard University Medical School, Boston.*)

PLATES 1 AND 2.

(Received for publication, March 26, 1928.)

The work to be described in this paper is the outgrowth of an observation (1) made in this laboratory a few years ago to the effect that the intradermal inoculation of rabbits with Type I pneumococci produces a characteristic and gradually extending lesion which, representing a sort of "dermal pneumonia," permits direct observation of changes, progressive or healing, under various experimental conditions.

Preliminary experiments showed that this lesion and the various occurrences as nearly resemble the sequences in human lobar pneumonia as do those of any other experimental method (except possibly experimental pneumonia in monkeys). Furthermore, the advantages of a visible lesion and the reliability in its production have led us to believe the technic to be a valuable one.

The production in animals of lesions comparable to lobar pneumonia has been attempted by many investigators. Wadsworth (2) produced typical lobar pneumonia by introducing pneumococci into the respiratory passages of partially immunized rabbits. Many others have employed this technic and modifications of it but such methods are not altogether reliable. Stillman and Branch (3) have produced inhalation pneumonias in mice and rabbits, but these pneumonias are not lobar and the results are not regular. Bull and McKee (4) have produced pneumonias in rabbits by intranasal inoculation. Lobar pneumonia in monkeys, such as that studied by Blake and Cecil (5), has some of the same disadvantages as the rabbit pneumonia and the expense of animals precludes their routine use.

The status of the pneumonia problem has not been materially changed since it was reviewed (1) in 1925. Human pneumococcus

lobar pneumonia is regarded as a symptom-complex arising from a local infection, complicated in many cases by the entrance of bacteria into the blood stream. Symptoms described clinically as those of toxemia are produced, although the exact cause of this condition is not known and no convincing demonstration of a pneumococcus toxin has been given. The therapeutic use of antisera as now prepared, even in concentrated form, has not proven satisfactory unless employed early in the course of the disease.

We have undertaken to resurvey the subject of pneumococcic infection, using the intradermal rabbit lesion as a basis for experimentation. In this paper we purpose to describe the lesion in detail and give a preliminary report on certain experiments bearing on immunity and serum therapy.

Experimental Methods.

Pneumococcus Cultures.—The Type I pneumococcus used in these experiments was grown in beef heart hormone broth (pH 7.5) to which small amounts of sterile defibrinated rabbit blood had been added. The virulence of this strain for mice was such that 0.000,000,01 to 0.000,000,001 cc. of an 18 hour culture, given intraperitoneally, sufficed to kill within 96 hours. This virulence was usually checked for each new experiment, the tests being made at the time of infecting the rabbits.

Rabbits.—Healthy, light colored female rabbits of 1300 to 2000 gm. were selected. The entire abdominal area of each animal was shaved, care being taken not to injure the skin in any way. The size, sex, or breed of the rabbit is unimportant but it must be of healthy stock. Because of the large amount of technical work involved, the largest convenient number of rabbits for a single experiment is six.

Infection.—After taking the necessary normal readings, the rabbit is given an intradermal inoculation of 0.2 cc. of the desired dilution of 18 hour broth culture. The site of infection is well up on the side of the shaved area, 6–7 cm. lateral to the rabbit's ventral midline.

Observations.—At regular intervals the appearance of the focal skin lesion is described, particularly as to its extent, the color of the various parts, the amount of swelling and the location of the excessively swollen areas, the consistency of the exudate, the amount of wrinkling and contraction of the skin, and the changes in these features from time to time.

Blood Cultures.—Using an accurately graduated tuberculin syringe a definite amount of blood is withdrawn from the rabbit's marginal ear vein. This blood and dilutions of it are plated immediately in cooled hormone agar. Small quantities of fresh, sterile, defibrinated horse blood are added in the case of dilutions. Plate counts obtained in this fashion represent approximately the number of

aggregations of pneumococci per cc. of circulating blood and give a rather accurate comparison of the septic processes at various times in different individuals. If carefully carried out, an almost indefinite number of such blood samples may be taken from the same animal.

Blood Samples Other Than for Cultures.—Since it is important to leave the marginal ear veins intact for obtaining blood for cultures it is necessary to obtain blood samples for other purposes from the smaller vessels of the ear. An area is carefully shaved and cleaned, and a small amount of xylol applied to the tip of the ear. When sufficient dilatation has been brought about, one of the smaller vessels is severed with a sharp razor, gross tissue injury being avoided. The blood flowing from the wound is collected and the serum used for the determination of content of protective substances, agglutinins, precipitins, etc.

Lesion Cultures.—The presence of pneumococci in the primary lesion is determined by culturing fluid aspirated from it. A fine gauge needle is inserted at a point previously treated with alcohol and a small amount of fluid is aspirated into a syringe and transferred to a blood agar plate. Positive results are regarded as more significant than negative for late in the disease, when the animal has lost considerable weight, it is often difficult to obtain fluid from the lesion.

The Focal Lesion Arising after Intradermal Infection.

The lesion usually makes its first appearance at 8 to 12 hours as an inflamed, swollen area 1 cm. in diameter. It progresses ventrally until at 24 hours it occupies a strip 2–3 cm. wide extending from the point of inoculation to, and across, the midline. The area at the midline is especially swollen by the accumulation of serous or serofibrinous exudate. Any further progression is along the ventral midline and the entire midabdominal area may become involved. Fig. 1 shows a drawing of a lesion at 30 hours. In the following paragraphs a detailed description is given.

The time of appearance of the initial lesion is dependent upon the number of organisms injected. With the intradermal inoculation of about 0.001 cc. of an 18 hour broth culture, the injection bleb completely disappears within the first half hour. In 8–10 hours the blood vessels on the infected side show congestion, then in the immediate vicinity of the inoculation the congested vessels lose their sharp outlines and the skin over a 1–2 cm. area takes on a highly inflamed color and becomes swollen. As this development continues the edema is most prominent on the ventral border. The lesion then spreads ventrally, the heightened color being usually preceded by the accumulation of fluid. The amount of swelling and the actual or apparent movement of the serous exudate give the impression that a viscous, unorganized fluid is seeping or flowing ventrally from the force of gravity. At 14–16 hours the edematous area usually has extended to

the midline. More serous material accumulates in the subepidermal tissues until the entire area is puffy. At 24 hours the lesion usually has a width of not over 3 cm. (as measured along the midline) but in length it extends from the point of inoculation ventrally to the midline and passes this by 2–3 cm. Within the next 24 hours the lesion usually extends along the midline in both directions, although the zone of greatest swelling is still located at the midline ventral to the point of inoculation.

In untreated animals the color of the lesion varies from a pale to a moderately bright orange red. In treated animals the lesion becomes uniformly red after treatment and then the color fades completely.

Depending upon local trauma and upon factors affecting the cutaneous circulation, parts of the surface of the lesion may become necrotic. This necrosis is preceded by petechial hemorrhages, and the area then takes on a greenish hue. Seepage from such areas tends to deplete the total amount of fluid and in such cases secondary infection may occur.

TABLE I.

Distribution of Deaths by Days in a Series of Fifty-Six Untreated Cases.

	Day of death						Recovered
	2nd	3rd	4th	5th	6th	7th	
No. of rabbits	12	16	6	7	4	3	8
Percentage distribution	21.4	28.7	10.7	12.5	7.1	5.3	14.3

Within 30–40 hours, even in fatal infections, the skin contracts and folds, large with serofibrinous exudate, develop. The mechanism of this process of contraction probably represents the involvement of the underlying muscles. In one typical case the shaved area had an original length of 13 cm. At 24 hours after infection this was 8 cm., while by 48 hours it was only 5 cm. Owing to the shortening of the abdominal surface the rabbit usually assumes an abnormal posture, with the hind legs drawn up closely under the body.

There is no change in the local lesion before or at death, but with recovery a process of resolution is instituted which is characterized by epidermal desquamation and fading of color. In the course of several days the lesion then disappears completely, and except in the case of previous necrosis no scar remains.

The Disease Other Than in the Focus.

The development of the focal lesion is accompanied by an abrupt rise in temperature and this temperature persists at a high level until

death or recovery. Organisms enter the blood stream within 12 to 24 hours.

The majority of untreated animals die with severe pneumococcus bacteriemia, the distribution of deaths by days being shown in Table I. At autopsy virulent organisms are found in all parts of the body. Save for the skin lesion and a slight enlargement of the spleen there is no significant pathology.

About 14 per cent of untreated cases recover spontaneously on the 5th, 6th, or 7th days. This recovery is preceded by the disappearance of organisms except from the focal lesion and is associated with an abrupt fall in temperature, changes in circulating cellular elements, the appearance in the blood stream of "protective substances," and with changes in the local lesion.

The relationship of these various occurrences to each other is described below.

The Bacteriemia.

In most instances, shortly after the appearance of the local lesion, organisms may be detected in small number in the circulating blood. This number tends to increase so that at 24 hours the aggregations of pneumococci per cc. of circulating blood in the average animal is about 50. In exceptional cases the number is so high as to grossly distort any arithmetical average of the entire series. The number of circulating organisms tends to increase gradually; this rate is not in strict geometrical progression such as would be expected if the bacteria were multiplying in the blood stream. In the typical case the number increases up to a certain point (near 100,000 per cc.) that appears to constitute a lethal level. The time necessary for arriving at this level may be as short as 30 hours but it is more often 60 to 70 hours after infection. In some animals there occur fluctuations in the bacteriemia such that at times no organisms may be isolated. These individuals are apt to ultimately recover. If the lethal level has not been reached in the characteristic period, recovery is associated with or preceded by the disappearance of the organisms from the blood stream.

Charts showing the development of the bacteriemia will be found below.

The Temperature Reaction.

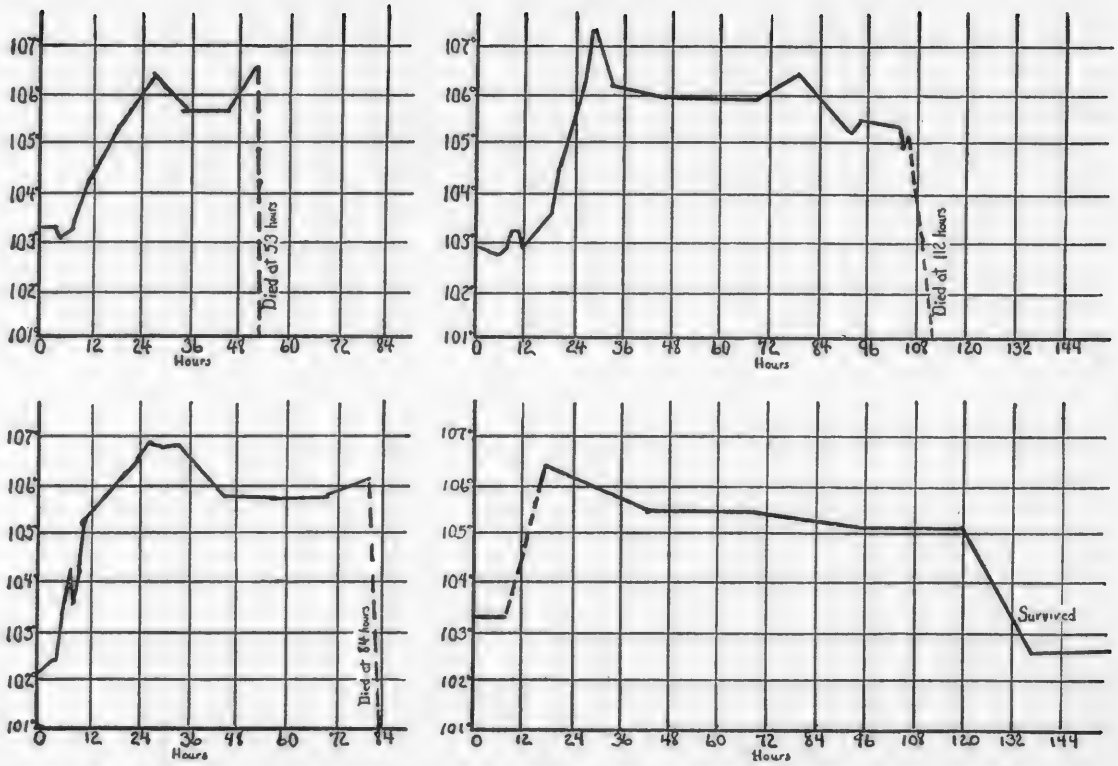
The temperature of the normal rabbit is variable and easily influenced by environment. In a series of 120 rabbits we find temperatures distributed over a range from 101.9° to 103.5°F., with an average of 102.8°F. The temperature of an acutely diseased rabbit undergoes less fluctuation due to environment and is of some significance when correlated with other data. Temperatures over 104.0°F. are regarded as definitely above normal levels.

At 5 to 12 hours after intradermal pneumococcus infection an animal's temperature begins to rise slowly and then abruptly goes up to a high level, usually over 105.5°F. The time occupied in this abrupt rise is ordinarily not over 4 hours. The third phrase is again one of slowly increasing temperature, a maximum being reached in 18 to 24 hours. This maximum ranges from 105.7° to 107.5°F. Then in spite of handling, the temperature remains at a high, though not stationary, level until death or the beginning of the recovery phase. In the case of death the temperature usually remains high until a few minutes before actual death.

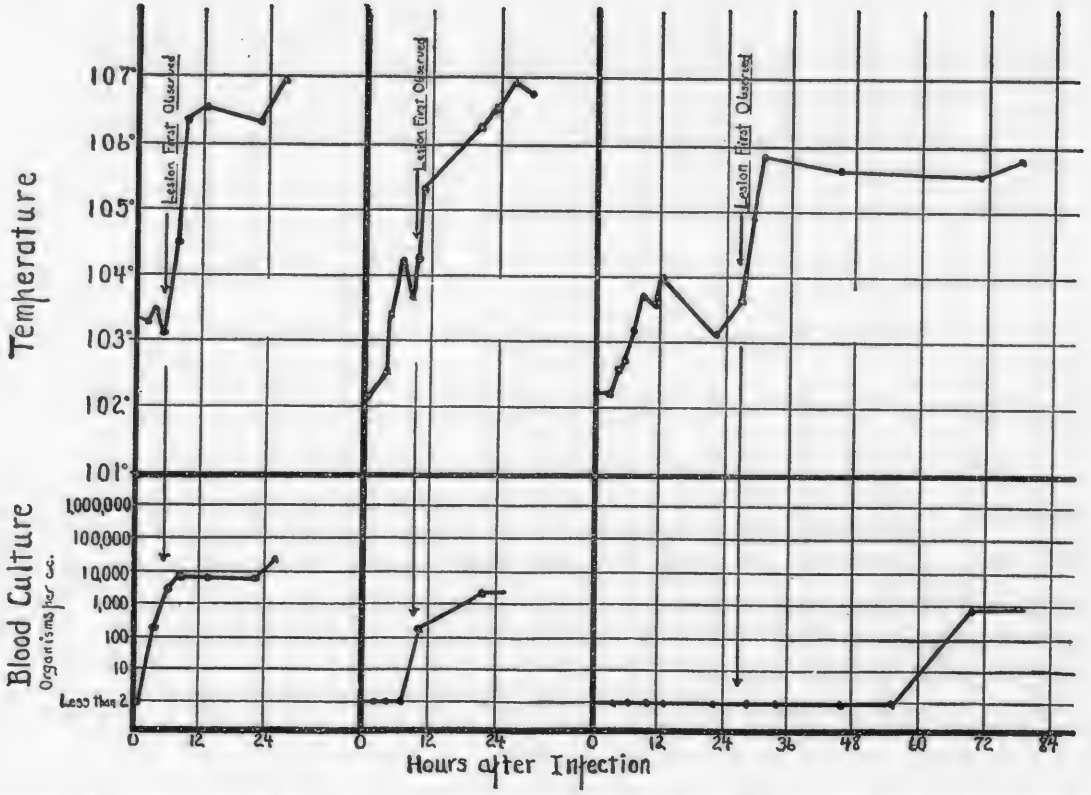
In the cases of rabbits which recover, the temperature persists at a high level for 5 to 7 days and then without warning drops to normal. This fall in temperature is rapid and is associated with other signs of recovery, in these respects not unlike what is spoken of as crisis in human pneumonia, another observation which persuades us of the close analogy of our rabbit disease with that of human pneumonia. As will be seen in a later section, again as in human disease, such a "crisis" is definitely correlated to the appearance of protective substances in the blood.

Text-fig. 1 shows charts of characteristic temperature curves in untreated cases.

It has long been believed that the temperature in lobar pneumonia is not related to the septicemia, but it seemed desirable to investigate this point in the experimental condition of the rabbit. An examination of records of a series of over fifty untreated cases shows that the temperature onset may occur (1) at the time when the organisms are first found in the blood stream, and more often this is the case; (2) it may occur as long as 36 hours before the beginning of the bacteriemia



TEXT-FIG. 1. Characteristic temperature curves in various rabbits, including one instance of survival without treatment. Infection in each case at 0 hour.



TEXT-FIG. 2. Temperature and bacteriemia determinations on three rabbits. These curves show the independence of the beginning of the bacteriemia and the onset of temperature These three cases reading from left to right represent maximal, average, and minimal intradermal dosage of pneumococci.

(as with minimal dosage); or (3) it may be delayed until several hours after the onset of septicemia (as with maximal dosage). Charts of examples of such findings are given in Text-fig. 2. The temperature onset is therefore independent of the bacteriemia.

From correlations of the appearance of the local lesion with the temperature it is apparent that the temperature rise always occurs exactly at the time that the local lesion is first observed.

The stimulus which brings about the continued febrile reaction is not easily determined. During this phase there are always living organisms in the local lesion, though not necessarily in the blood stream. The level of the temperature during this period is not proportional to the severity of the bacteriemia, nor is it proportional to the intensity of local inflammation, if the latter may be judged by color and edema. During this phase of high temperature the local lesion may undergo shrinkage or necrosis and it may become somewhat solid, but it never shows the cardinal signs associated with recovery: the appearance of surface desquamation and loss of inflammatory color.

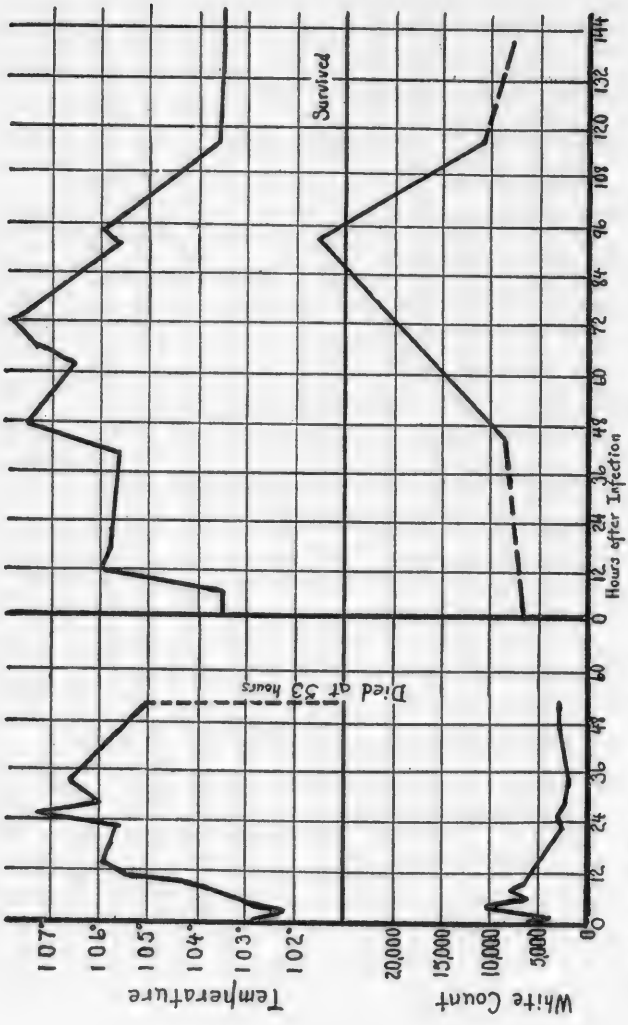
The sudden recovery, as marked by the fall in temperature, is not accompanied by the immediate disappearance of virulent organisms from the lesion, but in spite of this the lesion does show the above mentioned recessive signs.

In partially immunized rabbits, the detailed description of which will be found in a later paper, it is possible to give an intradermal injection of pneumococci and obtain a local inflammation. In this case of partial immunity there is little or no increase in temperature.

It seems therefore that the systemic effects indicated by temperature are not correlated with the presence of living organisms either in the blood stream or lesion but with the absorption from the focus, where living organisms are present, of something which either is no longer formed in the recovering or partially immune animal or which being formed is neutralized. We are led from this to believe that in the local lesion there is elaborated some toxic factor which in turn is responsible in large measure for the intensity of the general reaction.

The White Cell Count.

The white count on the rabbit is generally regarded as subject to great fluctuation. The average count in one series of fifty-four



TEXT-FIG. 3. Temperature and white count curves in two typical cases, one of which survived.

rabbits was 7280, but repeated counts on individual rabbits undergoing handling showed that it was difficult to establish a relative normal for any individual.

The first symptoms following intracutaneous infection are accompanied by a definite leucopenia, the decrease being accomplished largely by the disappearance of polymorphonuclear cells from the blood stream. In animals which recover, this leucopenia persists for 2 or 3 days and is followed by a definite leucocytosis, the number of white cells becoming normal again after the animal's recovery. In the case of animals which die during the course of the disease, the white count usually remains below normal throughout. The white count thus constitutes a definite prognostic sign, though it is by no means infallible. In Text-fig. 3 are shown examples of counts on each type of animal.

In many cases the number of white cells is roughly inversely proportional to the bacteriemia. With organisms circulating in the blood stream the white count is usually low but when the blood is freed of pneumococci the white cell count rises. There is some evidence to indicate that these inverse changes in white count do not accompany changes in the status of bacteriemia but occur only after such a change.

These findings are consistent with the favorable prognostic significance attributed in lobar pneumonia both to the absence of septicemia and to the high white count.

Lesion Cultures.

Cultures of fluids aspirated from the focal lesion in normal rabbits show large numbers of pneumococci during the active stages of the infection. Technical difficulties and late consolidation of the lesion sometimes prevent successful aspirations. At the time of recovery, as marked by various signs, there is a decrease in the number of organisms in the lesion, but virulent organisms may sometimes be isolated as long as a week and certainly for 1 or 2 days after crisis, which again is analogous to the presence of pneumococci in the lungs after crisis as demonstrated by Thomas and Parker (6).

Weight Loss.

One-fifth to one-fourth of the total body weight of the infected rabbit may be lost during the period of high temperature. A definite loss of at least 10 per cent has been an invariable finding. Some of this is undoubtedly due to the failure of the animal to take food but the emaciation initiated during the disease usually persists for several weeks after recovery, in spite of special diet and care.

Development of Protective Substances and Agglutinins.

The spontaneous recovery of untreated typical cases is associated with the appearance in the blood serum of protective substances. By this term we refer to that property of serum by which mice are passively protected against pneumococcic infection, provided that the serum is administered just prior to or at the time of infection. In such instances of critical recovery we have found that the precritical serum possesses no protective property but that serum which is immediately postcritical is protective against at least 0.1 cc. of highly virulent culture. This matter will be reported at greater length in a later paper and evidence will be presented to show that this point (immediately postcritical) may be the zone of greatest passive protective value of the serum.

Agglutinins and precipitins have never been detected in less than 2 days after recovery in the untreated animal. The agglutinins usually attain their highest titer at 12 to 18 days after infective inoculation (5 to 15 days after recovery).

The serum of convalescent rabbits is an effective therapeutic agent in the treatment of other cases. This will be reported on in detail in a later paper.

Pathological Examination.

At postmortem examination the skin at the point of greatest involvement is found to have a thickness varying from 12 to 18 mm., as contrasted to a normal thickness of 2 to 3 mm. On sectioning such a lesion one observes a subepidermal jelly-like mass from which straw colored fluid oozes. The skin at the point of original inoculation is usually slightly if at all thickened.

There is no constant gross pathology besides the skin lesion. In many cases there is a slight enlargement of the spleen but other organs are essentially negative. No perforations between lesion and peritoneum have been observed. It is possible to isolate virulent pneumococci from heart's blood, the serous cavities, and almost all tissues.

Microscopically, sections of the skin lesion show that the epidermis is not involved but that the corium is enormously swollen with serofibrinous material containing numerous cells. There is marked congestion of blood vessels and lymphatics. The subcutaneous connective tissues are usually swollen and here as well as in the muscle layers there is some cellular infiltration. In some cases there is a considerable atrophy of bundles of muscle fibers. Although fluid aspirated from the lesion during life reveals large numbers of diplococci, it is somewhat difficult to demonstrate these in sections. However it is certain that in the untreated lesion the organisms are extracellular. Fig. 2 shows a very low magnification of a cross-section of a pneumococcic skin. Fig. 3 represents a highly magnified detail from such a section. The character of the phlegmon is shown and in one portion there is an injected lymphatic.

The lungs are frequently congested but show no signs of pneumonitis. Virulent pneumococci may be isolated from the meninges post mortem but there is no meningitis.

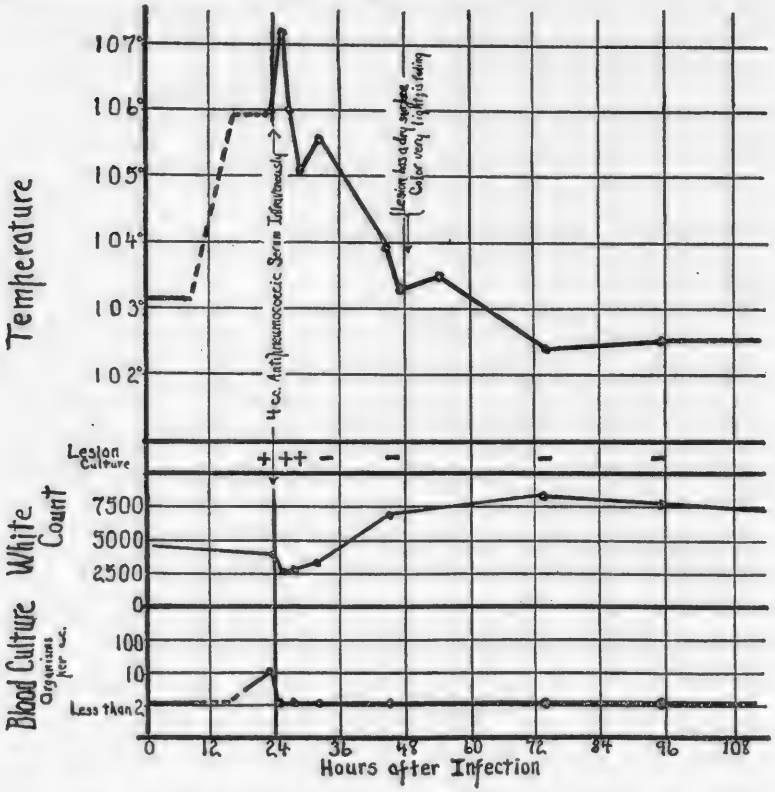
The Susceptibility of the Rabbit to Minimal Intradermal Infections.

It is generally recognized that the normal rabbit has no resistance against the pneumococcus but opinions differ as to quantitative infectivity: as to whether the necessary infective dose is a function of the weight of the animal, or of the number of virulent organisms in the culture employed.

We have found that the minimal quantity of culture necessary to infect mice intraperitoneally represents also the minimal quantity for intradermal infection of rabbits, that is to say, if a culture has a virulence for mice such that 0.000,000,1 cc. causes infection and death, the same amount constitutes the minimal intradermal dose for rabbits. This result is independent of the weight of the animal—that is, the necessary dosage is not the number of organisms per kilo of body weight but rather of organisms per individual rabbit. With smaller doses the onset of symptoms is delayed to such an extent that it is evident that the number of organisms must multiply *in vivo* to a certain level before an actual lesion is brought about. The disease resulting from a small dose, though delayed in first appearance, is in no way different from that caused by a larger infecting dose.

When undiluted virulent culture is injected intradermally there occurs as in other cases a definite latent period and then, perhaps 2 hours before the temperature rise and the actual appearance of the lesion, it is possible to isolate organisms from the blood stream. It

might be argued that the effective level was exceeded at the time of infection but that this did not hasten the termination of the latent period necessary for signs of local inflammation.

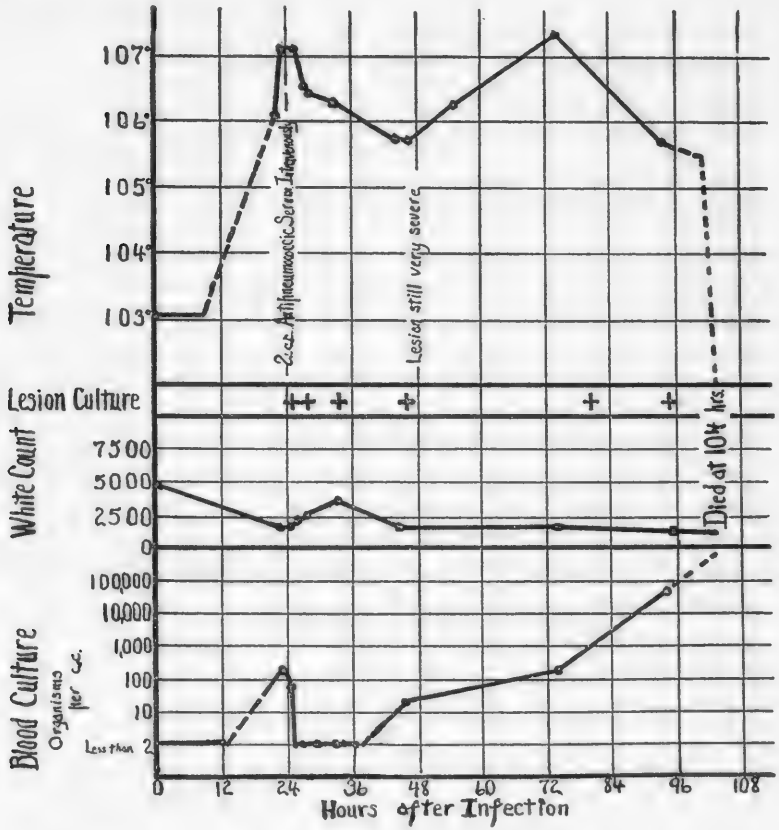


TEXT-FIG. 4. This chart shows the results obtained by treatment of a typical case with an amount of serum (4 cc.) which represents an excess over the M.E.D. (3 cc. for this serum). Weight of rabbit 1500 gm. Time of treatment, 24 hours.

Response of Infected Rabbits to Specific Serum Therapy.

Early in the course of this study, we began to experiment with various types of therapy. Since the intradermal infection of the rabbit represented a rather definite clinical symptom-complex, it was

reasoned that it might prove adaptable for (1) an analysis of the therapeutic action of specific sera, (2) for comparing the therapeutic values of various types of sera, and (3) for a method of standardization based on therapeutic value.



TEXT-FIG. 5. This chart shows the effect of treatment with an insufficient amount of serum. 2.0 cc. of a serum having a M.E.D. of 3.0 cc. were given at 24 hours. The only decided result is the temporary clearing of organisms from the blood stream.

We have not employed local therapy extensively, for, although antipneumococcic serum has a definite local protective effect in skin

areas in rabbits, it appears to have little influence if injected directly into an already active lesion.

Preliminary experiments demonstrated that prompt recovery could be brought about by the intravenous injection of a relatively large quantity of antipneumococcic serum provided that such injection be given early in the course of the disease. Further experiments were undertaken with the view of analysis of this therapeutic effect.

Text-fig. 4 shows the results obtained in a typical case by the intravenous injection at 24 hours following infection of a large amount (4 cc.) of antipneumococcic horse serum. The essential features of this therapeutic effect are: (1) the organisms immediately disappear from the blood and do not reappear, (2) the temperature begins to fall within a few hours and reaches a normal level within 24 hours, (3) organisms cannot be cultured from the local lesion after a period of a few hours following treatment, and (4) the skin lesion at first takes on a brighter color and then this color diminishes so that at 24 hours the area is very pale. At this time epidermal desquamation is also in evidence.

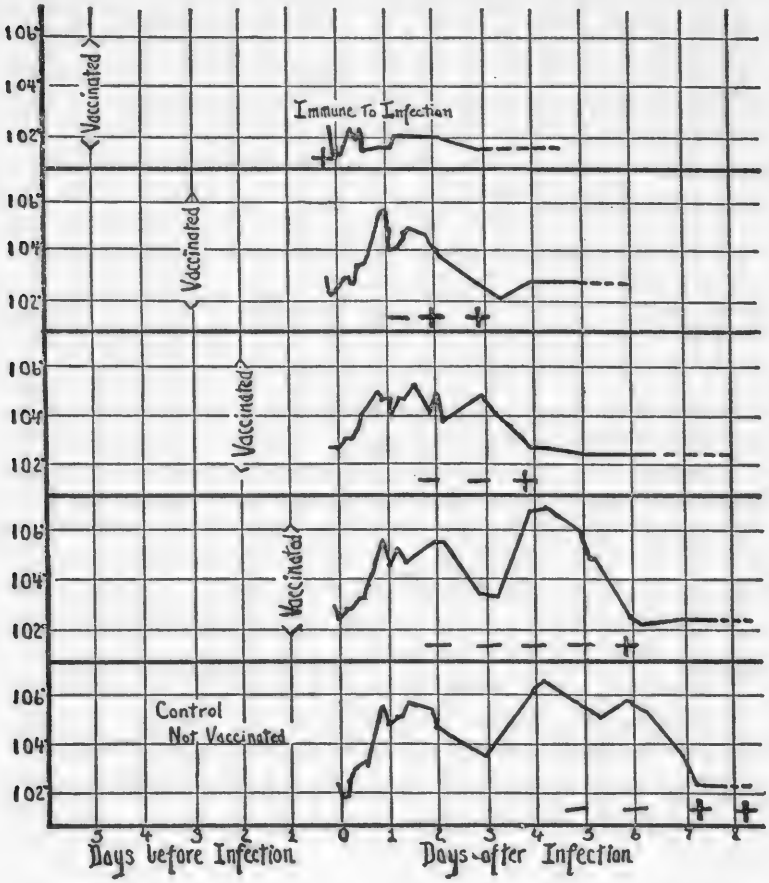
Experiments with a series of rabbits and graduated doses of serum show that beyond a certain point the excess of serum adds no value to that result which might be obtained with a definite minimum, this minimum being described as the minimal amount necessary to bring about the four decisive results given above. This definite value for any one serum is a relatively fixed quantity, other factors, such as time of treatment, being the same. This amount we designate as the minimal effective dose (M.E.D.).

If smaller amounts are administered it is found, depending upon the amount of serum, that there may be a temporary clearing of pneumococci from the blood stream but that this is not a permanent disappearance. In some instances (with doses that approach the M.E.D.) there may be a slight temporary depression of temperature and a considerable reduction in the bacterial content of the focal lesion. A chart of such a case is given in Text-fig. 5.

Observations on Active Immunization.

In the course of our work we have begun to apply this method of observation to the study of active immunization. While these studies

are being extended it is interesting in connection with the work reported above to note at this time the effects of a single injection of



TEXT-FIG. 6. Temperature charts on a series of five rabbits, four of which had been vaccinated at various intervals prior to infection intradermally at 0 hour. In each case at the time of crisis the usual recessive features were noted in the local lesion. + and − indicate presence and absence of protective substances.

dead pneumococci on the development of the local lesion, the course of the disease, and especially the development of the sudden drop of

temperature which we have spoken of as the "crisis." This is illustrated in an experiment the results of which are shown in Text-fig. 6.

Text-fig. 6 indicates: (1) animals vaccinated by one injection of dead pneumococci 5 days before infection develop neither lesion nor general disease and protective substances are present on the 5th day after vaccination, (2) animals vaccinated 3 days before develop a definite, but rapidly resolving lesion and recover by crisis, again on the 5th day after vaccination. The protective bodies appeared at this time, and (3) with each day by which the interval between vaccination and infection is shortened the recovery by crisis and production of protective bodies are prolonged by 1 day (in some cases slightly more than 1 day).

It appears therefore that one injection of vaccine is followed with regularity in 5 or 6 days by a condition of immunity sufficient either to prevent infection, or, if infection precedes the lapse of this period, to bring about recovery within approximately 5 or 6 days after the administration of the dead bacteria.

Inference from this in regard to the possible usefulness of vaccine administration to cases of influenza or measles or in the early stages of pneumonia are obvious but will necessitate further study.

DISCUSSION.

The detailed discussion of these findings must await the results of experiments now being undertaken.

The chief value of this experimental condition is that it offers in a suitable laboratory animal a symptom-complex which is in many respects analogous to human lobar pneumonia and has the additional advantages of a visible and easily produced lesion. With this condition it has been possible to correlate some of the features involved in the local lesion and such more generalized factors as the febrile reaction, the bacteriemia, changes in leucocyte count, the development of protective substances, etc. While many of these correlations might have been predicted from our knowledge of lobar pneumonia, it nevertheless furnishes additional evidence of the similarity of this "dermal pneumonia" to that of true pneumonia.

Of particular interest in this condition is the temperature reaction; its features are an abrupt onset, the high level maintained during the

course of the disease, and the abrupt or critical fall. It has been shown that the febrile reaction is not primarily a factor of the bacteriemia but possibly of some unknown substance elaborated and absorbed from the active local lesion. Evidence is presented to show that the temperature onset coincides with the first appearance of the local lesion and that this temperature persists for a rather definite period of time quite independently of changes in the status of bacteriemia.

It has been shown that the critical fall in temperature is associated with the appearance of protective substances. We do not know what part such substances have in the actual recovery for it is certain that they do not appear before or during the temperature fall but do appear in maximum titer immediately after crisis. Such a crisis is preceded by a disappearance of organisms from the blood stream but not necessarily by their disappearance from the local lesion.

Changes in white cell count indicate that the leucocytes play a passive part in the disease in an untreated animal and that the polymorphonuclear elements are low in number during bacteriemia and conversely. The white count constitutes a definite prognostic sign in this respect only.

With this method it is possible to analyse carefully the result of therapy and to compare the effects from various therapeutic agencies. We have reported our observations in cases treated with the ordinary antipneumococcic horse serum. The study of passive immunization is being continued and we are now developing in detail a method for standardization of the therapeutic properties of serum, based on the observation as to the minimal effective dose for any one serum. It is hoped that such a method may offer a more satisfactory basis for measurement of therapeutic value than the present mouse protection titration.

SUMMARY.

1. The intradermal inoculation of rabbits with Type I pneumococci gives rise to a local lesion and a definite sequence of other events that offer many analogies to pneumococcus lobar pneumonia, and for this reason the condition is being employed for a resurvey of the subject of pneumococcic infection.

2. This symptom-complex has been described in detail particularly as to the development of the local lesion, the temperature reaction, the bacteriemia, the white count, the persistence of organisms in the lesion, and the development of protective substances.

3. A certain number of animals recover from this condition after a definite course and by a process spoken of as crisis. The events correlated with this crisis have been described.

4. If sufficient antipneumococcic serum is given intravenously at 24 hours, prompt recovery can be brought about. The essential points of this recovery have been established.

5. Within 5 days after a single vaccination with dead pneumococci the normal rabbit develops an immunity to infection. If the rabbit is vaccinated and then infected within the period necessary for the development of this immunity the course of the consequent disease is shortened in proportion to the interval between vaccination and infection.

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EXPLANATION OF PLATES.

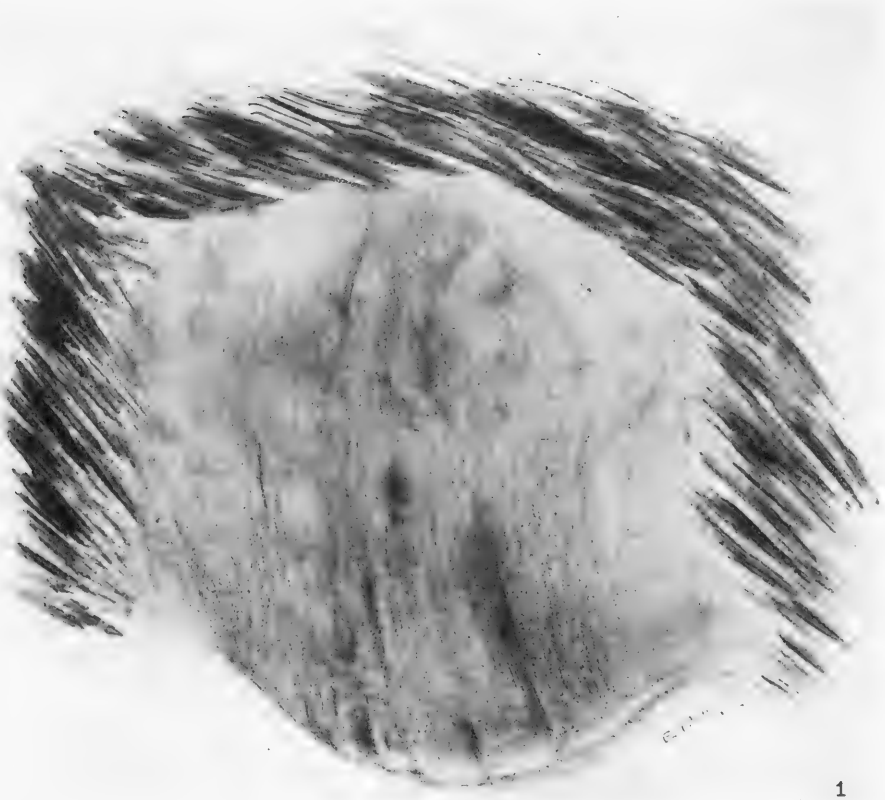
PLATE 1.

FIG. 1. Drawing of a characteristic lesion at 30 hours following intradermal infection.

PLATE 2.

FIG. 2. A photograph of a cross-section of pneumococcic rabbit skin; low magnification. The original cross-dimension of this skin was 13.5 mm.

FIG. 3. Highly magnified detail of the section shown in Fig. 2. The structure of the phlegmon is shown, and at the upper right there is an injected lymphatic.



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(Goodner: Intradermal pneumococcus infection.)

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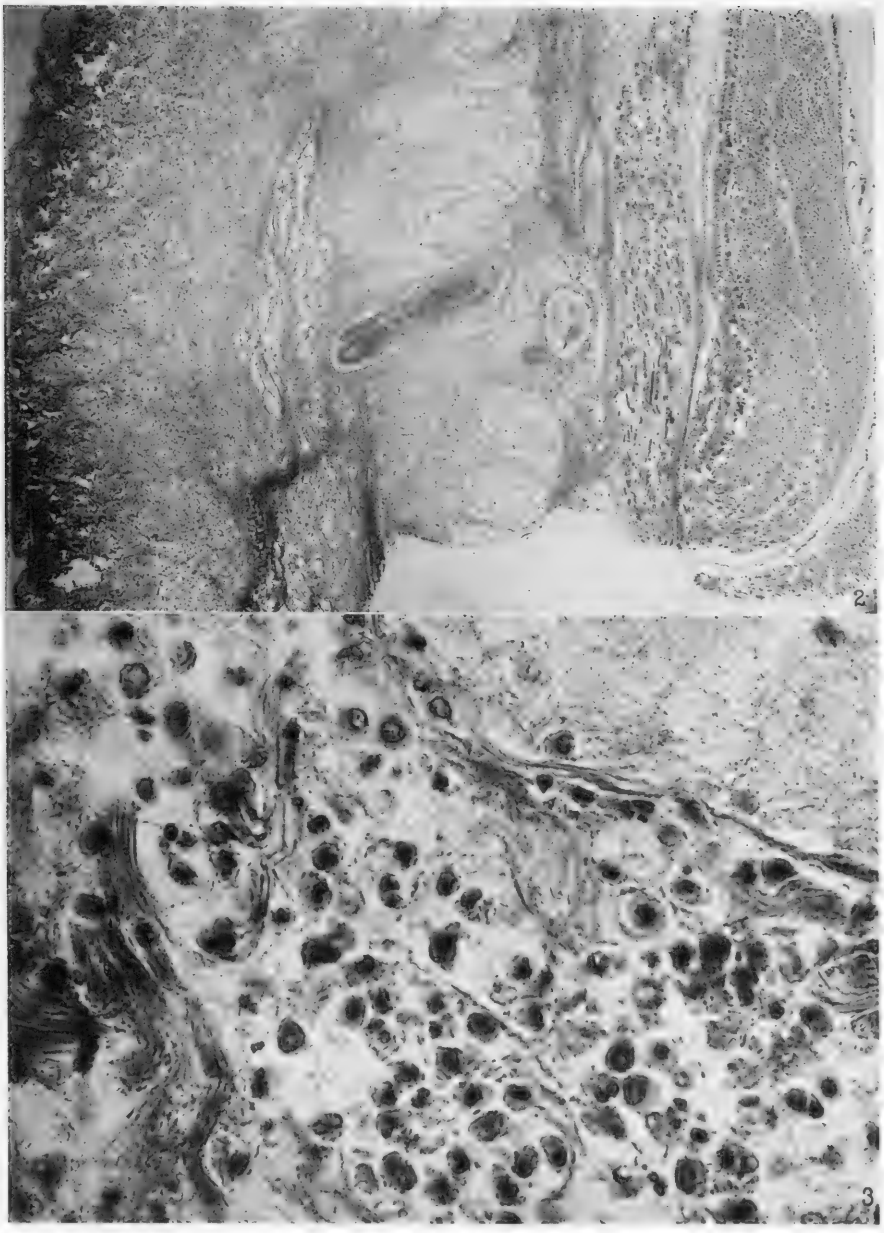

(Goodner: Intradermal pneumococcus infection.)

CHOLESTEROL AND CHOLESTEROL ESTER CONTENT OF BOVINE COLOSTRUM.

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(Received for publication, March 28, 1928.)

Colostrum, the first milk secreted in a lactation period is distinguished by the quantitative relationship of many constituents from the milk of later lactation. It is richer in the albumin and globulin fractions of the protein and in the salts. It contains many cells frequently spoken of as milk corpuscles which contain nuclear material. The melting point of its fat is higher. Fox and Gardner (1) and Dorlencourt and Palfy (6) working with human milk have observed that that secreted early in lactation is richer in cholesterol than that secreted late in lactation.

In so far as the recent literature refers abundantly to the relationship existing between cholesterol or related compounds and vitamine D, this possible change in the cholesterol content from colostrum to milk is of particular interest in its relation to the antirachitic and growth functions associated with colostrum or milk. In conjunction with investigations designed to test the validity of this viewpoint, colostrum was drawn from three pure bred Jersey cows at 12 hour intervals, commencing at 12 hours after parturition. The sample of colostrum was a true sample of all the colostrum secreted, as the calf was removed from the dam before suckling, and colostrum was drawn only at these intervals when the cow was milked dry. Two of the cows, Nos. 232 and 235, were being treated with ultra-violet light and No. 237 was not. The animals were all protected from direct sunlight. The later samples of milk were obtained in the 4th and 8th months of lactation. Cholesterol was determined by the method of Bloor (2) for blood using double quantities of milk and double quantities of the alcohol ether extractive. Bloor and Knudson's method (3) was used in determining cholesterol bound as ester modifying it in the same way as for the total cholesterol analyses.

DISCUSSION.

The main fact brought out by this investigation is the large proportion of cholesterol in the first colostrum drawn as contrasted with that secreted in the next 12 hours. The decline in the proportion of cholesterol secreted continues at an ever decreasing rate for the suc-

TABLE I.

Cow No.	Time after parturition that sample was drawn	Weight of sample	Total cholesterol	Cholesterol ester	
		kg.	*mg. per cent*	*mg. per cent*	*per cent*
235	12 hrs.		89	25	28
	24 "		53	14	27
Received ultra-violet light	36 "		34	7	19
	48 "		25	4	18
	Normal milk sample				
	3 mos.		17	6	36
	7 " a.m.		21	11	51
	7 " p.m.		23	6	25
232	12 hrs.	5.09	82	24	30
	24 "	5.72	38	8	22
Received ultra-violet light	36 "	7.54	36	7	20
	48 "	6.00	29	8	26
	Normal milk sample				
	3 mos.		19	4	25
	7 " a.m.		23	8	36
	7 " p.m.		23	3	13
237	12 hrs.	7.36	61	5	9
	24 "	3.50	46	5	11
Received no ultra-violet light	36 "	5.00	33	8	23
	48 "	5.09	29	4	15
	Normal milk sample				
	3 mos.		28	8	30
	7 " a.m.		19	12	64
	7 " p.m.		22	4	19

ceeding three periods of 12 hours each, when it reaches a level but little above that of the milk at 3 months. This decline in the proportion of cholesterol represents also a true quantitative decline as the weights of the 12 hour colostrum samples remain fairly constant. The level of cholesterol secretion is fairly constant for milk, as shown by compar-

ing the cholesterol in the milk at 3 and at 7 months. The significance of this decline in cholesterol is mathematically attested by the fact that Z is 1.6, while for $P = 0.05$, Z is only 0.6.* If this general trend in the cholesterol is compared with that of the other constituents in the colostrum, it is noted that the decline follows closely the secretion rates of the albuminous constituents up to the 48 hour period (4). After this time the albumin still continues to decline in value whereas the cholesterol remains fairly constant. A similar but less comparable decline in percentage amount occurs in the ash of the milk. The fat, casein and lactose show, if anything, a reverse tendency, rising in percentage rather than declining as lactation advances.

The total cholesterol per cent secreted by the cows receiving the ultra-violet light is larger than for the cow not receiving ultra-violet light for the first 12 hour period. Mathematically speaking, the difference is only suggestive, as the difference between ultra-violet and normal cows is less than 3 times its probable error.

The ester cholesterol of the colostrum appears to be quite variable. Cow 235 shows a decline comparable with that found for the total cholesterol. The other cows, especially for the 24, 36 and 48 hour periods, do not show the decline. The cows receiving ultra-violet light show slightly larger ester values than the cow not receiving light.

The morning and night variation of the cholesterol in 7 months milk indicates that normal Jersey milk contains about 22 mg. per cent of cholesterol. The amount of this total cholesterol bound as ester is twice as much in the morning milk as in the evening milk. This difference is significant as the value of P between the morning and night series is 0.03.

SUMMARY.

The total amount of cholesterol found in colostrum and milk is comparatively low. The amount of cholesterol found in colostrum declines at an ever decreasing rate as milk secretion develops until at 48 hours the cholesterol is nearly the same as that found in milk 3 months or 7 months after parturition. The morning milk differs from the evening milk in that the cholesterol bound as ester is greater in amount.

* In calculating the significance of the decrease found against the normal variation, P is a direct measure of the significance of the data obtained. Z is a calculated constant from which P or probability is obtained (5).

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CHOLESTEROL AND CHOLESTEROL ESTER IN THE BLOOD SERUM OF CATTLE LATE IN PREGNANCY AND DURING THE EARLY LACTATION PERIOD.

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(Received for publication, March 28, 1928.)

INTRODUCTION.

Chauffard, Laroche and Grigaut (1), Pribram (2) and others have observed that in the human being there is an increase in the cholesterol content of the blood throughout the latter half of pregnancy. After parturition and coincident with lactation a decrease in blood cholesterol results. The findings of Parhon and Parhon (3) on ducks and chickens indicate that in birds in the period just preceding laying there is an increase in the cholesterol content of the blood. During the egg-laying period there is a decrease in blood cholesterol. Baumann and Holly (4), using rabbits, found that, in pregnant animals, the concentration of cholesterol in the blood decreases from the 15th to 20th day after conception to reach the lowest values 1 to 4 days before parturition. The values rise again, irrespective of lactation, to the non-pregnant figures in 4 weeks after parturition.

The research herein reported was initiated for two reasons; the contrasting nature of the results on humans and birds as compared with those on rabbits, and the fact, which our previous investigations established (5), that the cholesterol in the colostrum period of mammary gland secretion is richest for the first secretion drawn, rapidly declining, as secretion continues, to a fairly constant level in the milk. The question therefore interests us as to whether these changes are due to an increase in blood cholesterol, or whether they are due to deposits of cholesterol in the udder during the dry period, or, third, to a greater secretory activity of the gland for this substance as contrasted with the other materials making up colostrum.

TABLE I.

Cow No.	Date and days in relation to parturition	Total serum cholesterol	Serum cholesterol ester	
	1927	*mg. per cent*	*mg. per cent*	*per cent*
45	Mar. 26 (30 days ante partum)	152	66	44
	Apr. 7 (18 " " ")	149	81	54
	Apr. 16 (9 " " ")	113	68	60
	Apr. 25, calved			
	Apr. 27 (2 days post partum)	100	54	54
	May 2 (7 " " ")	120	75	63
	May 6 (11 " " ")	114	84	74
	May 11 (16 " " ")	141	97	69
	May 16 (21 " " ")	162	102	63
	June 7 (43 " " ")	203	Lost	
	June 17 (53 " " ")	170	139	82
	June 27 (63 " " ")	211	119	56
141	Mar. 17 (39 days ante partum)	87	45	51
	Mar. 26 (30 " " ")	93	53	58
	Apr. 7 (18 " " ")	98	64	65
	Apr. 16 (9 " " ")	94	71	75
	Apr. 25, calved			
	Apr. 27 (2 days post partum)	94	54	51
	May 2 (7 " " ")	121	61	50
	May 6 (11 " " ")	122	77	63
	May 11 (16 " " ")	127	76	60
	May 16 (21 " " ")	119	83	70
	June 7 (43 " " ")	174	Lost	
	June 17 (53 " " ")	187	134	72
	June 27 (63 " " ")	181	139	77
47	Mar. 17 (32 days ante partum)	110	64	58
	Mar. 26 (23 " " ")	130	68	52
	Apr. 7 (11 " " ")	130	77	59
	Apr. 16 (2 " " ")	123	85	69
	Apr. 18, calved			
	Apr. 20 (2 days post partum)	104	76	73
	Apr. 25 (7 " " ")	114	76	67
	Apr. 30 (12 " " ")	117	79	68
	May 6 (18 " " ")	133	90	67
	May 11 (23 " " ")	130	102	78
	May 16 (28 " " ")	152	110	72
	June 7 (50 " " ")	175	Lost	
	June 17 (60 " " ")	153	133	87
	June 27 (70 " " ")	183	89	48

TABLE I—*Concluded.*

Cow No.	Date and days in relation to parturition	Total serum cholesterol	Serum cholesterol ester	
	1927	*mg. per cent*	*mg. per cent*	*per cent*
185	Mar. 17 (30 days ante partum)	163	96	59
	Mar. 26 (21 " " ")	139	95	68
	Apr. 7 (9 " " ")	130	93	71
	Apr. 16, calved			
	Apr. 18 (2 days post partum)	139	41	29
	Apr. 22 (6 " " ")	133	93	69
	Apr. 26 (10 " " ")	125	79	63
	Apr. 30 (14 " " ")	119	79	66
	May 6 (20 " " ")	125	93	74
	May 11 (25 " " ")	125	96	76
	May 16 (30 " " ")	133	76	57
	June 7 (52 " " ")	192	Lost	
	June 17 (62 " " ")	189	149	79
	June 27 (72 " " ")	189	107	56

EXPERIMENTAL.

Blood from four cows, 240, 241, 247 and 252 days pregnant, was obtained. The animals were bled from the jugular vein at intervals of a few days as given in Table I. Serum cholesterol was determined by the method of Bloor (6), and cholesterol ester by the method of Bloor and Knudson (7). The results obtained are given in Table I.

DISCUSSION.

Three very definite alterations occur in the cholesterol and cholesterol ester content of cow serum late in pregnancy and during the ensuing lactation period. First, the non-lactating period of late pregnancy is characterized by a serum low in both cholesterol and cholesterol ester. Secondly, 2 days post partum there is a further decrease in serum cholesterol ester and usually also in total serum cholesterol. Thirdly, during the early lactation period there is a gradual rise in both total serum cholesterol and serum cholesterol ester to reach a rather constant level sometime between 40 and 50 days post partum. The general trend of the serum cholesterol values is highly significant, mathematically speaking, as the value of Z is 0.97 where the value of

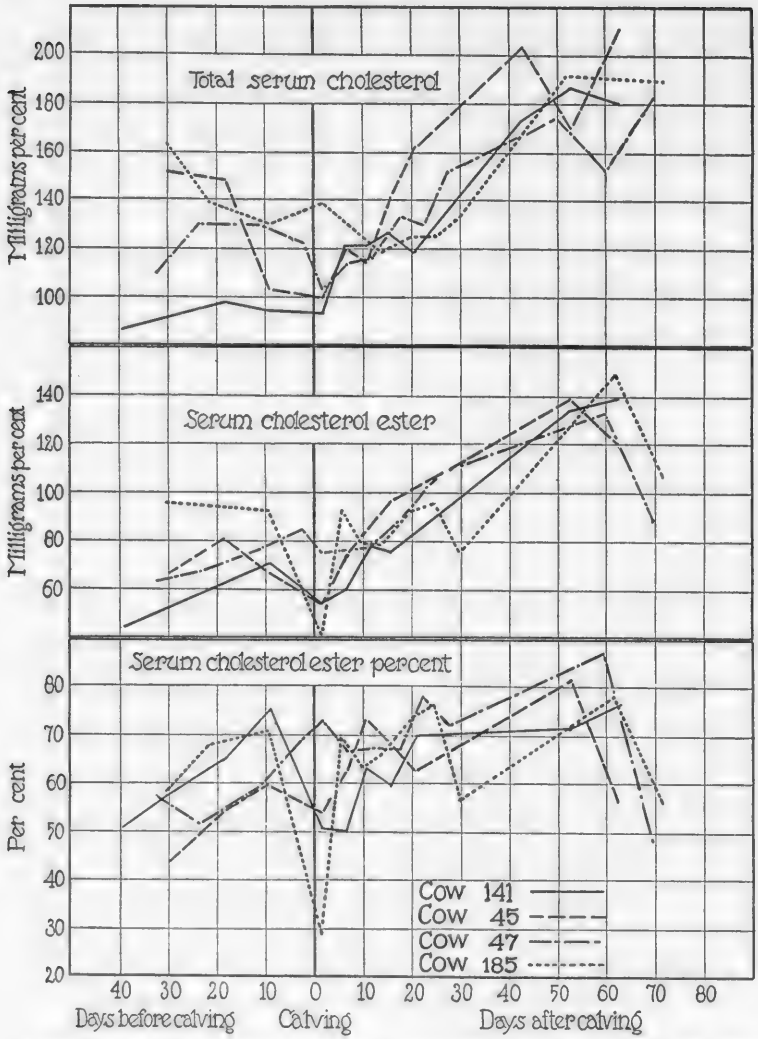

FIG. 1. Relation between pregnancy, parturition and lactation of the serum cholesterol and cholesterol ester in dairy cattle.

Z for $P = 0.05$ is only 0.39.* These changes may be most clearly visualized from Fig. 1.

There is some evidence tending to show a slight individuality in the serum cholesterol values of the cows making up the experiment. The value of Z for the four cows is 0.58, whereas that for $P = 0.05$ is 0.54, or the individuality of the cows in their serum cholesterol values is just over the border line of significance. The results therefore possibly show that the effect of physiological factors on serum cholesterol values is itself influenced by the individuality of the cow.

The cholesterol values of the blood serum as herein indicated show a trend opposite to that found for colostrum and milk as presented elsewhere (5), for instead of rising after parturition the cholesterol of colostrum and of milk declines. The cholesterol values of blood serum rise to a maximum at about 40 to 50 days. This rise corresponds in time to the rise taking place in the milk production and the attendant rise of the milk solids for most cows. It corresponds further to the period of more than ordinarily rapid drain on the body stored materials. This is particularly true of dairy cattle for which the aim of the breeder has always been the production of cows capable of a maximum milk yield.

The work herein described agrees with the facts found for rabbits.

SUMMARY AND CONCLUSIONS.

1. The total cholesterol and cholesterol ester of bovine blood serum during the "dry" period of late pregnancy is low.

2. Following parturition the total cholesterol and cholesterol ester of bovine blood serum show a still greater, very transient decrease followed by a gradual rise to reach a rather constant level 40 to 50 days post partum. This lactation level is very much higher than the "dry" level.

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INFLUENCE OF LIGHT ENVIRONMENT ON THE GROWTH AND NUTRITION OF NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

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Plate 3.

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It was shown in a previous paper (1) that the calcium and inorganic phosphorus in the blood of normal rabbits is subject to periodic variations and that among animals living in the laboratory there is also a tendency toward a progressive increase in calcium, as was first noted by Grant and Gates (2), and a more decided decrease in inorganic phosphorus of uncertain duration and extent with consequent variations in the equilibrium between the two substances. It appeared that several factors might contribute to the production of these variations among which were increasing age, adjustment to cage life, and changing conditions of light environment. The influence of light environment on the calcium and inorganic phosphorus content of the blood has been tested experimentally, but before proceeding with the analysis of the results obtained, it seems desirable to direct attention to some other observations which were made on the same animals, as these observations afford some interesting examples of the manner and extent to which functional activities and chemical equilibria may be affected by prolonged exposure to a light environment of a given type.

The object of the present paper is to report the results of a comparison of changes in body weight of normal rabbits living under different environmental conditions with especial reference to the action of neon light and to the existence of a relation between the nutritive status of an animal and the chemical composition of the blood under the conditions employed. This latter aspect of the subject will not be developed in this paper further than to point out a few instances in which there was some evidence of the occurrence of coordinate variations in body

weight and blood calcium. This phase of the problem will be presented in a subsequent paper.

Material and Methods.

The results to be reported in this paper represent one phase of a series of observations which were made on rabbits living under conditions which differed only with respect to the light environment. The observations made included records of weight, determinations of calcium and inorganic phosphorus from the same sample of blood, a study of the proliferative activity of hair follicles as indicated by the rate and extent of the regeneration of hair over shaved areas, and a comparison of the weights of organs at the conclusion of the experiments. This paper will be limited to a consideration of the results obtained for body weights with a brief reference to the blood findings in certain groups of animals.

The effects of three environmental conditions were compared. One group of animals, which may be designated as the controls, was kept in a well lighted (sunlight), well ventilated room with a southern exposure. The room was heated during cold weather and an effort was made to maintain a temperature of 60–70°F. (thermostatic regulation).

A second group of rabbits was kept in a room from which all light was excluded. Other living conditions were the same as those of the first group. During the course of the experimens, these animals received some light from two sources. For a brief period each day, a 30 watt Mazda lamp was used in this room for cleaning cages, feeding, and making the necessary observations. From time to time, the animals were also brought into the laboratory for bleeding and were thus exposed to diffuse filtered sunlight for a period which rarely exceeded an hour per week.

A third group of animals, the light group, was kept in a room similar to the others, with sunlight excluded. This room was lighted continuously by 20 standard neon tubular lights (crown glass), 6 feet in length and 5/8 inches in diameter. The tubes were mounted in an upright position on a rectangular frame in the center of the room, parallel with and facing the cages at a distance of approximately 3½ feet. The lights were operated on a current of 25 milliamperes at 8000 volts, to a bank of 3 lamps in series.

The light produced by neon Geissler tubes varies from a rose red to a bluish pink color depending upon the condition and the operation of the tube. The heat produced is very slight.

A spectrogram of neon light in crown glass is shown in Fig. 1, for which we are indebted to Dr. F. L. Gates. The rays transmitted are concentrated in two regions with faint bands intervening. The shortest rays lie between 3370 and 3620 Ångström units. The strongest of these are in the region of 3460 to 3480. The greater part of the light produced is of very long wave-length and is composed of rays from about 5800 to the lower limit of registration at 7600 Ångströms. The vertical illumination at the front of the cages varied between 21 or 23 foot candles at the ends of the rack and 54 at the center with a mean of 34.3 foot candles. No attempt was made to measure the energy delivered by this equipment

as it was perfectly obvious that such measurements would serve no particular purpose in the present instance.

We wish also to emphasize the fact that the animals used in these experiments were given no special preparation to facilitate the action of light such as clipping or shaving of hair.

Observations were made on three groups of normal male rabbits living under the conditions described. At the beginning of the experiments, the animals were 8 to 10 months old; they were sexually mature but had not attained their full growth. In each case, the animals for a given experiment were assembled and divided into three comparable subgroups according to type or breed, age, and weight. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage. They were weighed before being fed and, as far as possible, on the same day of each week throughout the period of observation.

Group I contained 15 albino rabbits, Group II, 15 black rabbits, and Group III, 15 gray, brown, or black rabbits. Groups I and II were placed under observation October 1, 1926 and were followed under control conditions until October 22; they were then placed in their respective experimental quarters and observations continued until May 18, 1927.

Observations on Group III began October 1, 1926. On February 8, 1927, the light and dark divisions of this group were interchanged so that animals which had been living in an environment of neon light for 4 months were placed in darkness and *vice versa*. This condition was maintained until May 4, 1927 when the two groups were again interchanged or restored to their original environment and observations continued until June 8, 1927.

The results obtained from the 3 experiments described have been supplemented by results from observations on two other groups of 10 rabbits each living under control conditions. These animals were grays and browns and were slightly younger than those in the regular experiments. The observations on one group (Group IV) began January 1, 1927 and on the other (Group V) March 1, 1927, and were continued, in both cases, until July 9, 1927.

The results of the study of body weight, as affected by light environment, are assembled in Tables I to XI. These tables contain mean values for the weight of given groups of animals at the time they were assembled, which is designated as the initial weight, and for consecutive periods of observation. In order to minimize the error due to unavoidable variations in the gastro-intestinal contents, the mean values have been smoothed by the formula $\frac{A + 2B + C}{4}$ and the smoothed value, rather than the actual mean, has been used as the basis of estimating gain or loss. The figures given for the gain in weight represent the difference between the initial weight and succes-

sive smoothed values. In order to reduce all expressions of weight relations to a comparable basis, the gain or loss has been converted into per cent of the initial weight, and these values are used for plotting the curves in Text-figs. 1 to 6.

RESULTS.

The results of the experiments are summarized in Tables I to XI and Text-figs. 1 to 6.

TABLE I.

Group I Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2319			
Oct. 7	2350	2327	8	0.34
" 23	2290	2321	2	0.09
" 30	2355	2358	39	1.68
Nov. 6	2430	2399	80	3.45
" 13	2380	2390	71	3.06
" 20	2370	2376	57	2.46
" 27	2385	2386	67	2.89
Dec. 4	2405	2396	77	3.32
" 11	2390	2394	75	3.23
" 18	2390	2393	74	3.19
" 29	2400	2433	114	4.92
1927				
Jan. 8	2540	2513	194	8.37
" 15	2570	2561	242	10.44
" 22	2565	2580	261	11.25
" 29	2620	2610	291	12.55
Feb. 5	2635	2623	314	13.54
" 11	2600	2608	289	12.46
" 19	2595	2589	270	11.64
" 25	2565	2563	244	10.52
Mar. 5	2525	2528	209	9.01
" 12	2495	2515	196	8.45
" 19	2545	2536	217	9.36
" 26	2560	2534	215	9.27
Apr. 2	2470	2468	149	6.43
" 9	2370	2453	134	5.78
" 16	2600	2550	231	9.96
" 23	2631	2619	300	12.94
" 29	2613	2625	306	13.20
May 7	2644	2644	325	14.01
" 14	2675	2666	347	14.96
" 19	2669			

TABLE II.

Group I Light—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	1955			
Oct. 7	1912	1964	9	0.46
" 23	2075	2054	99	5.06
" 30	2155	2139	184	9.41
Nov. 6	2170	2209	254	12.99
" 13	2340	2311	356	18.21
" 20	2395	2390	435	22.25
" 27	2430	2406	451	23.07
Dec. 4	2370	2408	453	23.17
" 11	2460	2433	478	24.45
" 18	2440	2465	510	26.09
" 29	2520	2479	524	26.80
1927				
Jan. 8	2435	2484	529	27.06
" 15	2545	2523	568	29.05
" 22	2565	2555	600	30.69
" 29	2545	2553	598	30.59
Feb. 5	2555	2558	603	30.84
" 11	2575	2599	644	32.94
" 19	2690	2660	705	36.06
" 25	2685	2694	739	37.80
Mar. 5	2715	2704	749	38.31
" 12	2700	2725	770	39.39
" 19	2785	2748	793	39.75
" 26	2720	2765	810	41.43
Apr. 2	2835	2780	825	42.20
" 9	2730	2764	809	40.55
" 16	2760	2781	826	42.25
" 23	2875	2840	885	45.27
" 29	2850	2850	895	45.78
May 7	2825	2835	883	45.17
" 14	2850	2833	878	44.91
" 19	2805			

TABLE III.

Group I Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2150			
Oct. 7	2160	2171	20	0.98
" 23	2215	2221	71	3.30
" 30	2295	2309	159	7.40
Nov. 6	2430	2380	230	10.70
" 13	2365	2403	253	11.77
" 20	2450	2375	225	10.47
" 27	2235	2315	165	7.67
Dec. 4	2340	2353	203	9.44
" 11	2495	2453	303	14.09
" 18	2480	2455	300	13.95
" 29	2363	2433	283	13.16
1927				
Jan. 8	2525	2443	293	13.63
" 15	2360	2408	258	12.00
" 22	2385	2363	213	9.91
" 29	2320	2380	230	10.70
Feb. 5	2495	2430	280	13.02
" 11	2410	2433	283	13.16
" 19	2415	2413	263	12.23
" 25	2410	2446	296	13.77
Mar. 5	2550	2484	334	15.53
" 12	2425	2483	333	15.49
" 19	2530	2498	348	16.19
" 26	2505	2509	359	16.70
Apr. 2	2495	2501	351	16.33
" 9	2510	2481	331	15.40
" 16	2410	2479	329	15.30
" 23	2585	2508	358	16.65
" 29	2450	2491	341	15.86
May 7	2480	2485	335	15.58
" 14	2530	2499	349	16.23
" 19	2455			

TABLE IV.

Group II Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2430			
Oct. 7	2480	2486	56	2.30
" 23	2555	2555	125	5.14
" 30	2628	2607	177	7.28
Nov. 6	2615	2606	176	7.24
" 13	2565	2585	155	6.38
" 20	2595	2596	166	6.83
" 27	2630	2624	194	7.98
Dec. 4	2640	2646	216	8.89
" 11	2675	2668	238	9.79
" 18	2680	2680	250	10.29
" 29	2685	2690	260	10.70
1927				
Jan. 8	2710	2710	280	11.52
" 15	2735	2756	326	13.42
" 22	2845	2825	395	16.26
" 29	2875	2865	435	17.90
Feb. 5	2865	2849	419	17.24
" 11	2790	2818	388	15.97
" 19	2825	2798	368	15.14
" 25	2750	2768	338	13.97
Mar. 5	2745	2745	315	12.96
" 12	2740	2756	326	13.42
" 19	2800	2784	354	14.57
" 26	2795	2794	364	14.98
Apr. 2	2785	2766	336	13.83
" 9	2700	2716	286	11.77
" 16	2680	2686	256	10.53
" 23	2685	2703	273	11.23
" 29	2760	2758	328	13.50
May 7	2825	2821	391	16.09
" 14	2875	2829	399	16.42
" 19	2740			

TABLE V.

Group II Light—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2405			
Oct. 7	2455	2426	21	0.87
" 23	2390	2425	20	0.83
" 30	2465	2465	60	2.49
Nov. 6	2540	2559	154	6.40
" 13	2690	2663	258	10.73
" 20	2730	2735	330	13.72
" 27	2790	2764	359	14.93
Dec. 4	2745	2795	390	16.22
" 11	2900	2878	473	19.67
" 18	2966	2957	552	22.95
" 29	2994	2946	541	22.49
1927				
Jan. 8	2830	2896	491	20.42
" 15	2930	2925	520	21.62
" 22	3010	2978	573	23.83
" 29	2961	2977	572	23.78
Feb. 5	2975	2978	573	23.83
" 11	3005	3023	618	25.70
" 19	3115	3083	678	28.19
" 25	3100	3105	700	29.11
Mar. 5	3105	3094	689	28.65
" 12	3065	3091	686	28.52
" 19	3130	3096	691	28.73
" 26	3060	3095	690	28.69
Apr. 2	3130	3098	693	28.81
" 9	3070	3081	676	28.11
" 16	3055	3086	681	28.32
" 23	3165	3129	724	30.10
" 29	3130	3134	729	30.31
May 7	3110	3135	730	30.35
" 14	3190	3150	745	30.98
" 19	3110			

TABLE VI.

Group II Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2100			
Oct. 7	2150	2134	34	1.62
" 23	2135	2151	51	2.43
" 30	2185	2206	106	5.05
Nov. 6	2320	2281	181	8.62
" 13	2300	2333	233	11.10
" 20	2410	2311	211	10.05
" 27	2125	2234	134	6.38
Dec. 4	2275	2269	169	8.05
" 11	2400	2365	265	12.62
" 18	2385	2385	285	13.57
" 29	2370	2393	293	13.95
1927				
Jan. 8	2445	2389	289	13.76
" 15	2295	2339	239	11.38
" 22	2320	2303	203	9.67
" 29	2275	2320	220	10.48
Feb. 5	2410	2365	265	12.62
" 11	2365	2373	273	13.00
" 19	2350	2346	246	11.71
" 25	2320	2360	260	12.38
Mar. 5	2450	2390	290	13.81
" 12	2340	2413	313	14.90
" 19	2520	2460	360	17.14
" 26	2460	2473	373	17.76
Apr. 2	2450	2448	348	16.57
" 9	2430	2414	314	14.95
" 16	2345	2393	293	13.95
" 23	2450	2405	305	14.52
" 29	2375	2404	304	14.48
May 7	2415	2418	318	15.14
" 14	2465	2436	336	16.00
" 19	2400			

TABLE VII.

Group III Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2200			
Oct. 15	2260	2315	115	5.23
Nov. 24	2540	2476	276	12.55
Dec. 29	2555	2558	358	16.27
1927				
Jan. 8	2580	2570	370	16.82
" 15	2565	2570	370	16.82
" 22	2570	2570	370	16.82
" 29	2575	2573	373	16.95
Feb. 5	2575	2581	381	17.39
" 11	2600	2603	400	18.18
" 19	2635	2635	435	19.77
" 25	2670	2678	478	21.73
Mar. 9	2735	2711	511	23.23
" 12	2705	2725	525	23.86
" 19	2755	2744	544	24.73
" 26	2760	2765	576	26.18
Apr. 2	2785	2780	580	26.36
" 9	2790	2791	591	26.86
" 16	2800	2806	606	27.55
" 23	2835	2820	620	28.18
" 30	2810	2789	589	26.77
May 7	2700	2738	538	24.45
" 14	2740	2751	551	25.05
" 18	2825	2796	596	27.09
" 25	2795	2779	579	26.32
June 3	2700	2723	523	23.77
" 8	2695			

TABLE VIII.

Group III Light—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2169			
Oct. 15	2288	2266	97	4.47
" 23	2319	2336	167	6.70
" 30	2419	2399	230	10.60
Nov. 6	2438	2488	319	14.71
" 13	2656	2603	434	20.01
" 20	2663	2672	503	23.19
" 27	2706	2694	525	24.20
Dec. 4	2700	2711	542	24.99
" 11	2738	2725	556	25.63
" 18	2725	2758	589	27.16
" 29	2844	2780	611	28.17
1927				
Jan. 8	2705	2770	601	27.71
" 15	2830	2815	646	29.78
" 22	2895	2873	704	32.46
" 29	2870	2881	712	32.83
Feb. 5	2890	2879	710	32.73
" 11	2965	2886	717	33.06
" 18	2925	2904	735	33.89
" 25	2900	2928	759	34.99
Mar. 5	2985	2944	775	35.73
" 12	2905	2969	800	36.88
" 19	3080	3041	872	40.20
" 26	3100	3074	905	41.72
Apr. 2	3015	3019	850	39.19
" 9	2945	2989	820	37.81
" 16	3050	2996	827	38.13
" 23	2940	2954	785	36.19
" 30	2885	2924	755	34.81
May 7	2985	2974	805	37.11
" 14	3040	3023	854	39.37
" 21	3025	3034	865	39.88
" 27	3045	3005	836	38.54
June 7	2905	2945	776	35.78
" 9	2925			

TABLE IX.

Group III Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2220			
Oct. 15	2220	2251	31	1.40
" 23	2345	2334	114	5.14
" 30	2425	2440	220	9.91
Nov. 6	2565	2533	313	14.10
" 13	2575	2580	360	16.22
" 20	2605	2568	348	15.68
" 27	2490	2533	313	14.10
Dec. 3	2545	2555	335	15.09
" 11	2640	2612	392	17.66
" 18	2625	2614	394	17.75
" 29	2565	2571	351	15.81
1927				
Jan. 8	2530	2566	346	15.59
" 15	2640	2595	375	16.89
" 22	2570	2584	365	16.40
" 29	2555	2581	361	16.26
Feb. 5	2645	2608	388	17.48
" 11	2585	2605	385	17.34
" 19	2610	2606	386	17.39
" 25	2620	2638	418	18.83
Mar. 5	2700	2686	466	20.99
" 12	2725	2726	506	22.79
" 19	2755	2746	526	23.69
" 26	2750	2768	548	24.68
Apr. 2	2815	2789	569	25.63
" 9	2775	2799	579	26.08
" 16	2830	2849	629	28.33
" 23	2960	2925	705	31.76
" 29	2950	2945	725	32.66
May 7	2920	2940	720	32.43
" 14	2970	2928	708	31.89
" 21	2850	2850	630	28.38
" 28	2730	2806	586	26,40
June 7	2915	2811	591	26.62
" 9	2685			

TABLE X.

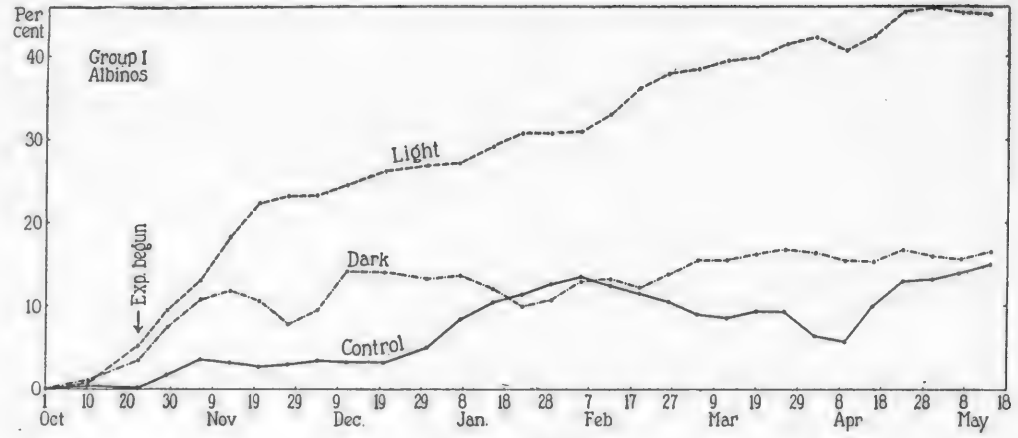
Group IV—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1927	*gm.*	*gm.*	*gm.*	*per cent*
Initial	1942			
Jan. 8	1961	1963	21	1.08
" 15	1986	1990	48	2.47
" 22	2028	2026	84	4.33
" 29	2061	2058	116	5.97
Feb. 5	2081	2085	143	7.36
" 11	2117	2127	185	9.53
" 19	2192	2166	224	11.53
" 25	2161	2182	240	12.36
Mar. 5	2213	2204	262	13.49
" 12	2230	2225	283	14.57
" 19	2225	2245	303	15.60
" 26	2300	2278	336	17.30
Apr. 2	2286	2288	346	17.82
" 9	2280	2266	324	16.68
" 16	2218	2244	302	15.55
" 23	2260	2255	313	16.12
" 29	2283	2289	347	17.87
May 7	2328	2331	389	20.03
" 14	2383	2362	420	21.63
" 27	2353	2362	420	21.63
June 3	2358	2337	395	20.34
" 18	2278	2329	387	19.93
" 25	2403	2359	417	21.47
July 1	2350	2377	435	22.40
" 9	2405			

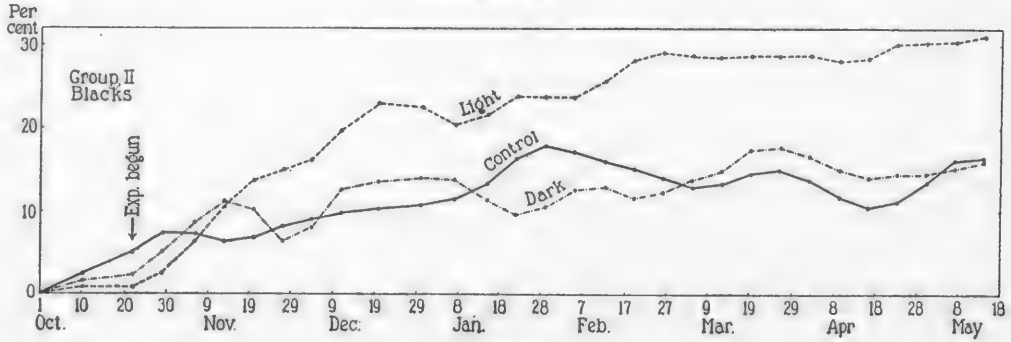
TABLE XI.

Group V—Body Weights.

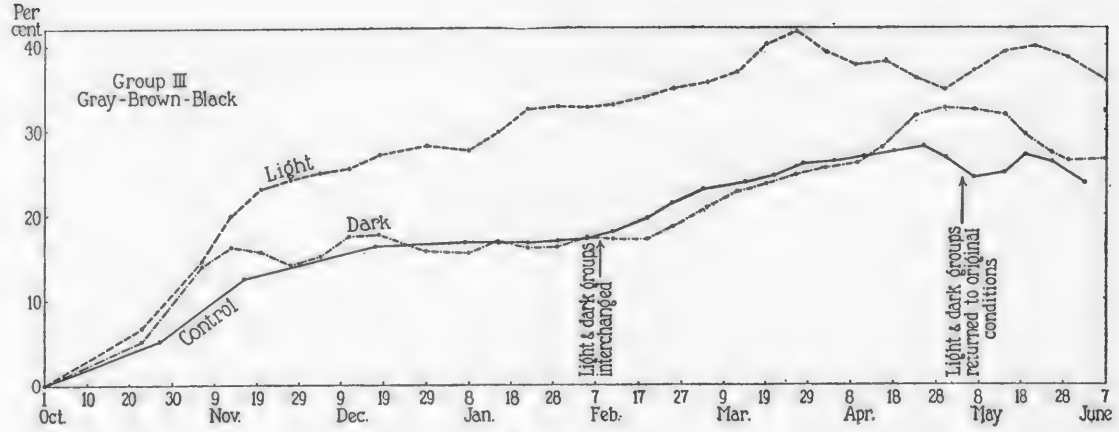
Date	Group mean	Smoothed mean	Net gain	Net gain
1927	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2075			
Mar. 23	2265	2201	126	6.07
Apr. 2	2198	2205	130	6.27
" 9	2160	2167	92	4.43
" 16	2150	2146	71	3.42
" 23	2125	2148	73	3.52
" 29	2190	2185	110	5.30
May 7	2223	2235	160	7.71
" 14	2283	2268	193	9.30
" 21	2273	2281	206	9.93
" 27	2295	2295	220	10.60
June 3	2315	2311	236	11.37
" 10	2320	2315	240	11.57
" 17	2305	2334	259	12.48
" 25	2405	2376	301	14.51
July 1	2388	2416	341	16.43
" 9	2482			



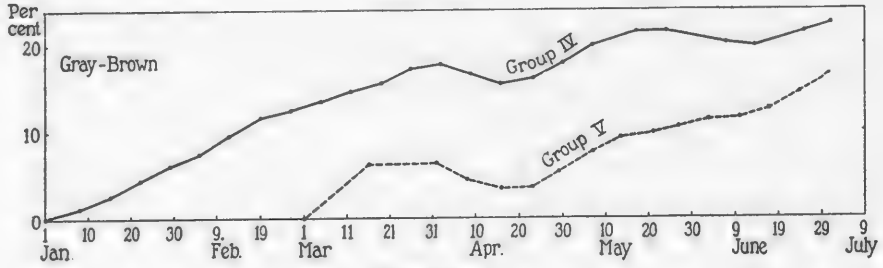
TEXT-FIG. 1.



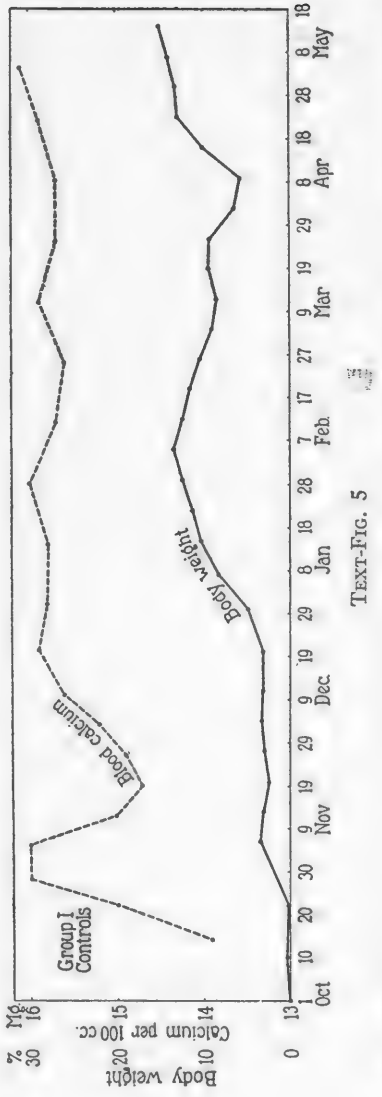
TEXT-FIG. 2.


Per cent
40
30
20
10
0
Group III
Gray-Brown-Black
Light
Dark
Control
Light & dark groups interchanged
Light & dark groups returned to original conditions
1 10 20 30 9 19 29 9 19 29 8 18 28 7 17 27 9 19 29 8 18 28 8 18 28 7
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June

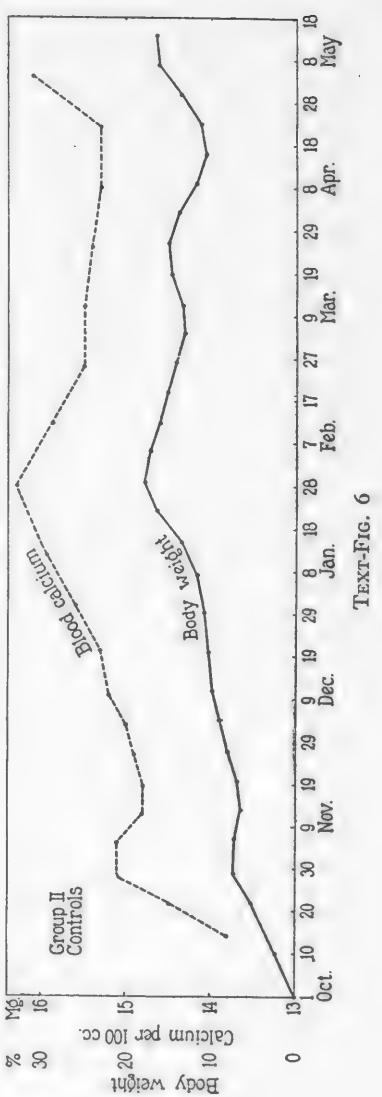
TEXT-FIG. 3.



TEXT-FIG. 4.



TEXT-FIG. 5



TEXT-FIG. 6

DISCUSSION AND CONCLUSIONS.

From an examination of the text-figures, it is evident that the animals exposed to neon light showed a greater gain in weight than those receiving diffuse, filtered sunlight or living in darkness. It should be noted also that of the three groups of rabbits living in an environment of neon light, the greatest gain was made by the white animals and that the black animals gained the least which is the reverse of the condition shown by control and dark groups. Theoretically, the black animals might be expected to show the greatest gain as the animals of this group were of a larger breed (Flemish crosses), and in our experience rabbits of this type gain more under the usual laboratory conditions than the common albino, as was true in the present instance. In view of these facts, the gain made by the white animals under the influence of neon light is especially significant.

The differences between groups may be brought out more clearly by a tabulation of the maximum gain shown by each group in per cent of the initial weight. For the purpose of this comparison, the results for Group III are divided. The first column gives the gain prior to the interchange of light and dark animals while the second gives the maximum gain irrespective of changes in environment. The results on this basis are as follows:

Gain in Weight According to Groups.

	Group I	Group II	Group III	
Light	45.78	30.98	32.73	41.72
Dark	16.70	17.76	17.66	32.43
Control	14.96	17.90	17.39	28.18

Viewed from this standpoint, it will be seen that the gain in weight of the albino rabbits (Group I) receiving neon light was approximately 3 times that of the dark and control divisions of this group; in the case of the black animals and the dark colored animals of Group III, it was nearly twice as great. A further point of interest that is brought out by this table is the agreement of the results for animals of comparable or different types under a given condition. Thus, if we consider the first results for Group III, the agreement between Groups II and III

is very close for a given environmental condition. These two groups are comparable as both were composed of dark colored animals, while Group I was distinctly different and the results are of a different order with the exception of animals living in the dark; the difference in this case is very slight as might be expected.

Considering the effects of the change in light environment of the animals of Group III, it will be seen by reference to Text-fig. 3 that at the time this change was made the weights of light, dark, and control animals were in a state of virtual equilibrium, and that following the change, the weights of all three groups began to increase at nearly the same rate. This situation was maintained for about 5 weeks when the animals that had been transferred from light to dark showed a more rapid increase for a brief period, followed by a decided loss in weight which continued up to the time they were returned to the light.

Meantime, the weights of the animals which had been transferred from dark to light continued to increase at a rate only slightly greater than that of the controls, making a gain of approximately 9.00 per cent during a period of about 2 months as compared with practically no gain during the 4 months preceding. Then as the weights of the light animals decreased, those of the dark animals increased at a more rapid rate—adding 6.50 per cent to their weight in 3 weeks, while the light animals lost 7.00 per cent. During this time, the weight of the controls also decreased. This appears to be a definite response to a change in environmental conditions.

The changes in weight which followed the return of the animals to their original environment are of uncertain significance. There was, however, an immediate increase in the weight of the light animals and a decrease in that of the dark group, but the changes that occurred were probably complicated by other factors, or by the persistence of effects of previous exposures to one or the other of the two environmental conditions.

In this connection, it is important to note that the response to the first change of light environment was not immediate. The gradual increase in weight shown by the animals transferred from darkness to light may have been a reaction to the change in the mode of living, and this possibility cannot be disregarded merely because the controls showed a similar reaction at the same time. Still, the first definite

evidence of a response was shown by the light animals in the form of a rather abrupt increase in weight followed by a prolonged decrease. This came 5 or 6 weeks after these animals had been placed in the dark. It was 2 months, however, after the dark animals had been placed in the light before the increase in weight of this group assumed a form that was clearly indicative of a reaction to the new environment.

A delay in the reaction to light or to the exclusion of light did not occur at the beginning of the experiments, when the animals were first placed in the light or dark, which suggests that the effect produced by these fixed environmental conditions involves more than a mere gain or loss in weight. The manner in which the metabolic activities of the animal are affected appears to be just as characteristic as the extent of the effect produced. Referring first to the curves in Text-figs. 1 to 4 which show the changes in weights of animals living in the open laboratory (control conditions), it will be seen that there is nothing constant or characteristic in the form of these curves. In some instances there is an immediate and progressive increase in weight continuing over a period of months with eventual stabilization and perhaps a further increase at a later date. This type of curve is shown by the animals of Group III (Text-fig. 3). In other instances, the gain in weight is delayed or there may be an initial loss or a very slow increase. Moreover, it will be seen that at certain times, as in midwinter, the increase in weight is greater than at others, or a maximum is attained while all animals are apt to lose weight at other times (spring and early fall). Unfortunately, no systematic observations are available for the summer months.

These peculiar fluctuations in weight are, of course, not confined to control animals, but they are most clearly defined among animals living in the open laboratory, and the loss of weight that occurs during the spring and fall is clearly associated with moulting. If the weight curves of control animals have a characteristic feature, it is the occurrence of these periodic increases and decreases in weight, and it will be noted, that animals brought into the laboratory at or near the same time show a closer agreement with respect to the occurrence of these fluctuations than animals that are brought in at different periods of the year. This will be seen by comparing the control curves in Text-

figs. 1 and 2 with those in Text-fig. 4; the curve in Text-fig. 3 is exceptional.

The weight curves of animals exposed to neon light or living in an environment from which light is excluded are distinctly different from those of control animals. In the first place, animals placed under either of these conditions showed an immediate and decided increase in weight; the initial rate of increase was much the same in the two cases, if allowance is made for possible differences in the composition of groups. In the case of light animals, the gain in weight was continuous and gradually developed into a series of step-like increases marked by brief periods of stabilization or reduction in the rate of gain which were succeeded in turn by more rapid increases. A definite decrease in weight was extremely rare, and so far as we have been able to determine, occurred only with some irregularity in feeding, as during or immediately following a holiday period, or in connection with moulting or the development of disease.

The weight curves of animals living in the dark are equally characteristic. One of the most distinctive features is the occurrence of a definite loss of weight following the gain of the 1st month or 6 weeks. This gain followed by a loss forms the characteristic pattern of the curve which is repeated over and over again with little or no material change in level. The general level is usually that of the control animals, but it will be seen that the fluctuations do not necessarily coincide with those of any other group of animals.

We have emphasized the distinctive features of the weight curves because it has been found that in certain instances variations in the chemical composition of the blood of animals living under these conditions give evidence of the occurrence of movements of a similar nature. It is not our intention to discuss this aspect of the influence of light environment at this time, but in order to indicate something of the nature of the relation that appears to exist, the results obtained for blood calcium on two groups of rabbits have been plotted against those for body weight (Text-figs. 5 and 6). The actual values for body weight and for blood calcium have been smoothed in the same manner, so that the curves are strictly comparable. The form agreement between these curves is so close that there can be no doubt that in these particular instances the variations in body weight and blood

calcium are related. It is evident, therefore, that conditions of living which affect one of these values may produce corresponding effects on the other in that a variation in one value may be reflected by the other. This is as far as this phase of the subject will be carried at present.

Others have found that confinement of animals in the dark for varying periods of time and irradiation by such means as the carbon arc or quartz mercury lamp will affect the rate of growth, or gain in weight, as well as the chemical composition of tissues and body fluids (3), but comparatively little attention has been given to fixed environmental conditions, or to a study of effects produced by prolonged exposure to light of long wave-length. It has been shown, however, that under appropriate conditions both the physical constitution or organic equilibrium of animals and the reaction to disease may be greatly affected by light of comparatively long wave-length such as that produced by Mazda lamps and the low pressure mercury arc in crown glass (4).

From the results obtained in these experiments, it is also evident that prolonged exposure to fixed environmental conditions, such as those represented by neon light or complete exclusion of light, may produce a profound effect on the animal organism (rabbit). In the present instance, the influence of environmental conditions was shown by a marked effect on the ultimate weight attained and also on the rate of gain and the form of the weight curve.

The increase in weight that occurs with animals of the ages used is attributable, in part to skeletal growth, in part to muscular development, and in part to an accumulation of fat. In general, the skeletal and muscular development, or growth in stature of animals exposed to neon light was greater than that of animals living in the dark; fat was also more abundant, but with few exceptions, animals living in the dark appeared to be well nourished and showed large accumulations of fat in all depots, even though the gain in weight had virtually ceased. In the one case, the effect produced suggests a definite promotion of growth with heightened metabolic activity while in the other, metabolic activity was apparently depressed or held in abeyance. Control animals differed from those living in a fixed environment in that they

tended to show prolonged periods during which one or the other of these conditions prevailed.

When animals were placed in the light or dark for the first time, the response was comparatively prompt, but with continued existence in the fixed environment, it appears that a persistent, or almost fixed, tendency was established so that the effect produced on body weight by a subsequent change from one environment to the other was greatly delayed. At first sight, it might appear that in this respect the effects of prolonged exposure to a fixed artificial environment differ from those of exposure to a natural light environment, but the difference may be more apparent than real as there is some evidence to indicate that tendencies established or accommodation to conditions that prevail during one period of the year, as in summer or winter, may persist for some time thereafter and modify the effects produced by a subsequent change in environmental conditions.

These results are of interest from still another point of view, namely, the denudation of body surfaces as a necessary means of obtaining effects from exposure to light. The effects produced may conceivably be increased by direct exposure of a skin surface, but these experiments show conclusively, as have others from these laboratories, that as a routine procedure, denudation is not necessary and in certain instances is undesirable as extensive denudation of body surfaces may give rise to conditions which will complicate the results of experiments.

In the present state of knowledge concerning the biological action of light, it is impossible to give a satisfactory explanation of the results obtained in these experiments. The present tendency is to correlate biological effects with the energy developed by rays of a given wave-length. From experiments carried out on this basis, it would appear that biological effects are confined very largely to rays in the ultra-violet region of the spectrum, or to rays with a wave-length of 3300 Ångström units or less. We wish to emphasize the fact, however, that there is a fundamental difference between a brief exposure of a living organism to light of given wave-lengths and prolonged existence in an environment of the same light; moreover, it is evident that results obtained under one set of conditions have no necessary bearing on the results that may be obtained under the other conditions. At-

tention may also be directed to the fact that results obtained by intense irradiation of experimental animals are usually complicated by a reaction to injury which makes it difficult to distinguish between that which represents a physiological response to radiant energy and that which represents a pathological reaction to the damage produced.

Our experiments deal with effects of light environment rather than irradiation. The results are clearly defined and are not complicated by a reaction to injury. For present purposes, it is immaterial whether the promotion of growth and nutrition that was observed among animals living in an environment of neon light is regarded as an effect of the small amount of short wave-length radiation or the larger amount of light of long wave-length. In either case, the illumination and the total energy involved are so small that they afford no basis for a satisfactory explanation of the effects produced. In all probability, the animals living under control conditions received a larger amount of light energy with higher illumination values than those exposed to the neon light, but in one case, the light environment was inconstant and subject to wide variation, while in the other it was virtually fixed. We regard this as an important factor. At any rate, it has been shown that, under certain conditions, differences in light environment which represent small amounts of light of comparatively long wave-length and small equivalents of energy are capable of producing clearly defined biological effects, and that the effects produced vary with the color or breed of the animal and are probably modified by still other factors, such as the age of the animal and the time at which the experiments are carried out.

SUMMARY.

The influence of light environment on the growth and nutrition of normal rabbits was studied by comparing the weight curves of animals living under different environmental conditions for periods of 4 to 8 months and the effects of change from one environment to another. Prolonged exposure to neon light was compared with confinement in the dark and exposure to diffuse, filtered sunlight of varying intensity.

The results of the experiments showed that growth and nutrition were greatly affected by the light environment in which the animals lived. The effects produced by a given environment varied with the

color or breed of the animal and appeared to be out of proportion to the differences in the intensity of the light or the energy represented.

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Neon Light

Exposure in seconds

80

40

20

10

5

2

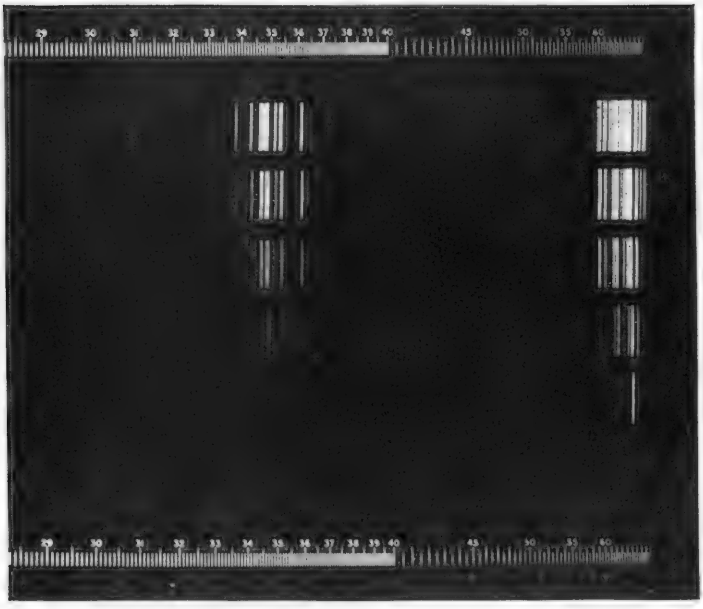

FIG. 1.

INFLUENCE OF LIGHT ENVIRONMENT ON THE GROWTH OF HAIR IN NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

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With the Collaboration of Marion Howard.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 23, 1928.)

In a previous paper,[1] it was shown that continuous exposure to neon light, or living in an environment from which all light was excluded produced definite and characteristic effects on the growth and nutrition of normal rabbits when compared with control animals living in the laboratory and receiving diffuse, filtered sunlight.

The influence of the 3 types of light environment on these animals was tested in still other ways. Parallel observations were made on the calcium and inorganic phosphorus content of the blood, and in the course of these investigations, it was noted that when the ears were shaved for bleeding, there was a striking difference in the growth of hair from one bleeding to the next. This difference in the growth of hair appeared to offer a satisfactory basis for a simple test of functional activity in the three groups of animals. At the time these experiments were in progress, Stevens was conducting a series of experiments in another laboratory of this Institute on the action of ultra-violet light and other agents on the growth of hair in rabbits, and the results obtained by him encouraged us to use the growth of hair over shaved areas as an index of cellular activity. The purpose of this paper is to report the results of the tests made.

In presenting these results, we wish to make it clear that it is not the purpose of this paper to discuss effects of light or of any other agent on the growth of hair as a problem in itself but merely to present the results of a series of experiments in which the growth of hair was used as an index of the proliferative activity of a particular group of cells.

[1] Brown, W. H., *J. Exp. Med.*, 1928, xlviii, 31.

Material and Methods.

The animals used for the experiments to be reported were the same as those used for the study of the influence of light on growth and nutrition,[1] so that a detailed statement of the general condition of the experiments need not be repeated.

The procedure employed in testing the proliferative activity of follicular cells was to compare the rate and extent of the growth of hair over a shaved area of the skin of rabbits that had been living in a given light environment for a long period of time. The conditions compared were continuous exposure to neon light, complete darkness, and a variable environment of diffuse, filtered sunlight. The tests were made as follows: the hair on one side was clipped and shaved over an area measuring approximately 8 × 5 cm. Care was taken to avoid trauma of any kind and to maintain uniformity in the location of the areas shaved and in the shaving itself.

The animals were examined at brief intervals throughout the experiments, first, for evidence of trauma, or of a traumatic reaction, and then for the purpose of determining the time at which growth of hair could be detected and the rate and extent of the growth that occurred until restoration was complete. The observations made on the growth of hair on the trunk were supplemented by observations of the same kind on one ear. In this case, a narrow band was shaved from the base to the tip of the ear along the course of the central vessels.

Three groups of 15 rabbits each were used for the tests. In each case there were 5 animals exposed to neon light, 5 in the dark, and 5 served as controls. Group I was composed of white rabbits, Group II of black, and Group III of gray, brown, and black animals. One experiment was carried out in midwinter when, normally, the proliferative activity of hair follicles is comparatively slight, and another at the time of the spring moult.

The animals of Group III were used for the first experiment. They were shaved December 29, 1926. At that time, they had been living in their respective environments for approximately 3 months. Observations were made under existing environmental conditions for 6 weeks. The light and dark groups were then interchanged and observations continued for 12 weeks after which the animals were restored to their original environments for an additional period of 4 weeks, giving a total observation period of 23 weeks (December 29, 1926, to June 8, 1927).

The animals of Groups I and II were used for the second experiment. They were shaved April 8, 1927 which was approximately 5½ months after they were placed in their experimental quarters (October 22, 1926, to April 8, 1927). The records of hair growth cover a period of 40 days.

RESULTS.

The results obtained for the growth of hair on the side are presented graphically in Figs. 1 and 2.

The rectangular areas in these charts represent the shaved area; the proliferation of follicles and growth of hair are shown by the shading. A clear rectangle means

no reaction; line shading in one direction indicates proliferation of hair follicles with a short or thin growth of hair; cross-hatching a fairly long or thick growth of hair, while solid black indicates complete restoration over the area thus marked.

The results obtained for the growth of hair on the ears are not presented as they were essentially the same as those for the trunk. The only difference noted was a somewhat more rapid growth of hair on the ears which may have been influenced to some extent by the occasional use of xylol in bleeding or by trauma. Neither of these factors affected the results for the trunk.

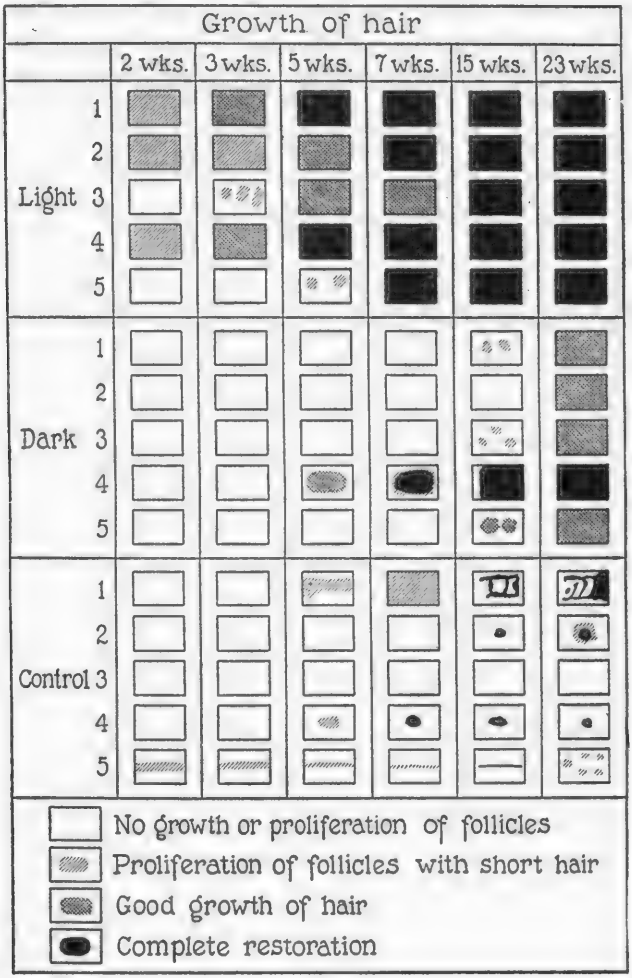

FIG. 1.

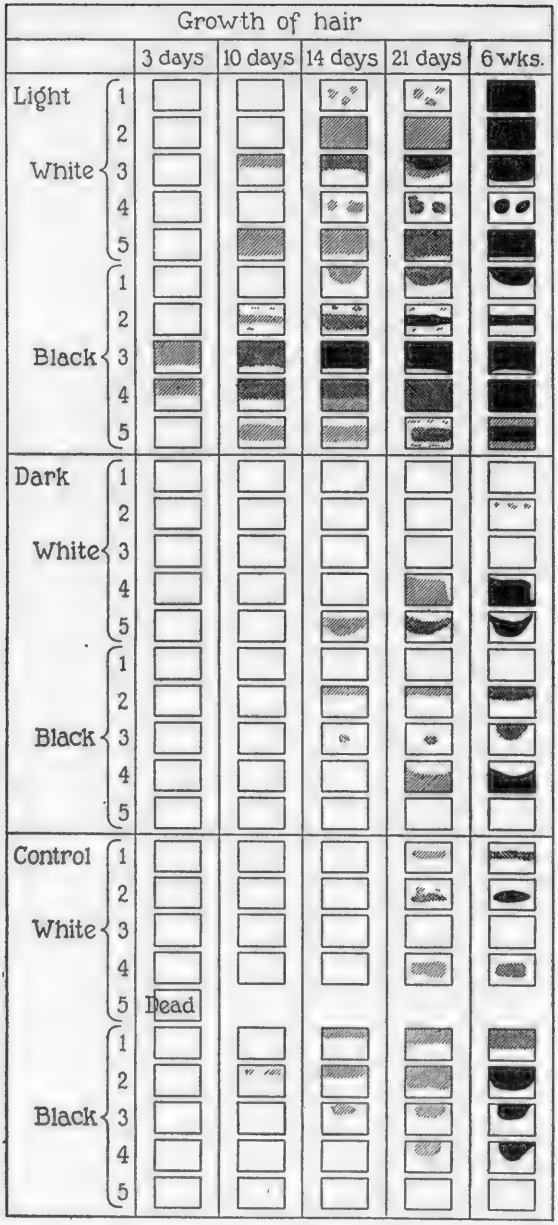
Growth of hair
3 days
10 days
14 days
21 days
6 wks.
Light
White
Black
Dark
White
Black
Control
White
Dead
Black
1
2
3
4
5

FIG. 2.

DISCUSSION AND CONCLUSIONS.

The difference in the response shown by animals living under different environmental conditions is very striking (Figs. 1 and 2). Within 5 to 10 days, 9 of the 15 rabbits that received neon light showed active proliferation of hair follicles with a definite outgrowth of hair, while among those in the dark there were none, and there were only 2 of the controls that gave any evidence of a reaction, and one of these (No. 5, Fig. 1) was not a true reaction. The narrow line of proliferating follicles seen in this animal was present at the time of shaving and did not increase thereafter; on the contrary, it diminished, indicating that the condition was merely an irregularity in the growth of hair such as is frequently seen among rabbits living in the laboratory. Differences of the order of those displayed by the three groups of animals during the first 2 weeks were maintained until the end of the experiments.

Comparing the results for the 2 experiments, it will be seen that among the animals living in an environment of neon light, the reaction was not quite as prompt in the first experiment (midwinter) as in the second (spring moult). In the second experiment, all animals showed an active growth of hair within 2 weeks, while in the first experiment, 2 of the 5 animals showed no reaction at a corresponding time and in one of these the growth of hair did not begin until towards the end of the 4th week. The end result was, however, essentially the same, but at the end of 6 weeks, restoration was more nearly complete in the first than in the second experiment.

Among control animals and those living in the dark, the differences between the 2 experiments were greater. None of the 15 control animals showed complete restoration of hair over the shaved area. In the first experiment, there was a fairly good growth of hair in 1 animal, but at the end of 23 weeks, this was still irregularly distributed. In the second experiment, the reaction was much more prompt and at the end of 3 weeks, 7 of the 9 animals showed a definite reaction as compared with none in the first, while at the end of 6 weeks, irregular patches of long hair were present in 4 of the 9 rabbits of the second experiment and none of the first.

A comparison of the results obtained for animals in the dark must take account of the fact that in the first experiment these animals

were transferred from darkness to an environment of neon light 6 weeks after they were shaved and remained in this environment for 12 weeks. The reverse change of environmental conditions did not affect the results for the light group, as the restoration of hair was virtually complete when the transfer was made. In the case of the dark group, however, there was a definite growth of hair in only one animal at the end of the first 6 weeks (a black rabbit); the skin of the others was as smooth and devoid of any evidence of reaction as though the hair had just been removed by some depilatory reagent. This condition was not changed in the slightest degree for 6 or 7 weeks. Scattered patches of proliferating follicles then appeared and gradually the hair began to grow; growth was not appreciably disturbed when the animals were again returned to the dark, so that at the end of the experiment, all of them showed a good growth of hair over practically the entire shaved area, but only 1 of them a complete restoration.

This result is especially important in comparison with the controls which showed a more active growth of hair during the first half of the experiment, but far less during the second half. Furthermore, beginning at about the 16th week, or 10 weeks after they were placed in the light, the growth of hair in the dark group was almost equal to that of the light group during the first 6 weeks of the experiment. This is also significant as it shows that the initial inactivity of the hair follicles of these animals was slowly abolished and replaced by a highly active condition. It is also of interest to note that the time required for the development of this condition in the skin is in fairly close agreement with the results obtained for the establishment of a definite increase in the rate of gain in weight.[1]

The results for the second experiment on animals living in the dark agree with those for the first in showing an initial state of comparative inactivity. The reaction, was however, much more prompt in the second than in the first experiment, and by the end of 6 weeks there were only 4 of the 10 rabbits that showed no reaction, while there were 2 animals in which the restoration of hair was complete over the greater part of the shaved area. In this experiment, the proliferation of hair follicles and the growth of new hair among the animals in the dark was almost equal to that of the controls, but still far less than that of animals in the light.

From a comparison of all results for the first and second experiments, it is evident that, irrespective of environmental conditions, there was a distinct difference in the promptness and constancy of the reaction displayed in the two cases. The difference between the two light groups was least as these animals, with few exceptions, showed an almost immediate response; the difference was greater between the two groups of controls with respect to the time, the constancy, and extent of the reaction, and still greater in the case of animals living in the dark. It is evident, therefore, that while complete exclusion of light may depress the proliferative activity of the follicle cells to a certain extent and exposure to neon light may increase their activity, in neither case is the effect such as to prevent or completely obscure the normal tendency to the occurrence of periods of heightened activity as at the time of moulting.

A further point of interest concerns the behavior of white as compared with black animals. It is not certain that a clearly defined difference in the reaction of the two classes of animals can be established, but there are suggestive differences. In the first place, the records show that in the light group of the second experiment (Fig. 2) the black rabbits reacted more promptly than the whites. This was true also for control, and probably for dark animals. At the conclusion of the experiment, however, restoration of hair over the shaved area was further advanced in the white than the black rabbits of the light group, while among the controls the situation was reversed with no decided difference in the dark group.

These differences are apparently slight and might be disregarded were it not for the fact that they agree absolutely with the results obtained for increase in body weight, and hence suggest that the two groups of functional reactions have a common basis which, in all probability, is to be found in an effect produced by the light environment on metabolic processes. This conclusion gains additional support from the parallelism between the effects produced on the growth of hair and the gain in weight of animals that were transferred from a dark to a light environment, as noted above. It is not to be inferred, however, that gain in weight and growth of hair are regarded as processes that are necessarily related, for, as was pointed out in a previous paper, a loss in weight is not infrequent at the time of moulting.

Summarizing the results of the experiments, it may be said that it was found that rabbits that had been living in an environment of neon light for a period of 3 months or more showed active proliferation of hair follicles and virtually complete restoration of hair over shaved areas within 5 to 8 weeks, while among animals living in the dark for the same length of time, or among controls receiving diffuse, filtered sunlight, the reaction was greatly delayed and, in the end, was irregular and incomplete. The growth of hair was, however, more active in all classes of animals during the spring than during the winter months, and there appeared to be a difference in the behavior of white and black animals.

It was also found that the skin of animals that were totally inactive after a prolonged existence in the dark became active after exposure to neon light for a period of 8 to 10 weeks.

As the results of these simple tests of the proliferative activity of the cells of hair follicles agree in every respect with those obtained for increase in body weight of the same animals, it is reasonably certain that the two sets of results have the same significance. Taken together, they show conclusively that conditions of light environment may have a profound influence on the animal organism (rabbit) and it is highly probable that an influence of some kind is exerted, irrespective of the wave-length or energy equivalent of the light concerned.

SUMMARY.

In a series of experiments dealing with the influence of light environment on normal rabbits, the growth of hair over shaved areas was used as an index of functional activity. The conditions compared were exposure to neon light, complete exclusion of light, and exposure to diffuse, filtered sunlight of varying intensity.

It was found that prolonged existence under these conditions affected the proliferative activity of hair follicles in a manner and to an extent comparable with the effects produced by the same environmental conditions on the growth and nutrition of the animals themselves.

RELAPSE PHENOMENA OF SPIRONEMA RECURRENTIS.

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Our present knowledge of the interaction of host and parasite in relapsing fever may be summarized as follows.

At the time of the crisis which terminates the attack of fever, there is rapid agglutination and destruction of the spirochetes with the subsequent formation of immune bodies in the blood (Sawtschenko and Melkich (1); Novy and Knapp (2)). These substances are specific for the strain of spirochetes which was present during the preceding attack, but have no influence on the spirochetes of the succeeding relapse. The spirochetes of the relapse give rise, in turn, to immune substances which are specific for them but not for the spirochetes of the first attack (Levaditi and Roché (3); Jansco (4)). Some workers have encountered, in successive relapses, several modifications of the original strain of spirochetes (Kudicke and Feldt (5)), while others have found only two alternating strains (Cunningham (6)). During epidemics of relapsing fever, some investigators have found a remarkable uniformity in the strain causing the first attack (Jansco (4)), while others have found several different strains (Toyoda (7)). Passage through its natural intermediate host, the tick, has also been shown to produce a change in the serological reactions of the spirochete (Kroo (8)). The new strains of spirochetes produced by relapses have been found by some workers to remain immunologically different from the spirochetes of the first attack through many animal passages (Levaditi and Roché (3); Cunningham (6)), while others have described a gradual reversion to the original strain (Kudicke and Feldt (5); Toyoda (11)). In mice which have apparently recovered from the infection, either naturally or by arsenical treatment, the brain and sometimes other organs are infectious for new mice for a period of several weeks (Buschke and Kroo, (9)), and spirochetes have actually been found in very small numbers in the blood stream of patients during the interval between attacks (Kudicke, Feldt and Collier, (10)).

Plan of Work.

The present study was undertaken for the purpose of studying further the changes in serological reactions produced during the

relapses of *Spironema recurrentis*. The animals used were the gray squirrel (*Sciurotamias davidianus*[1]) and the striped chipmunk (*Eutamias asiaticus*), both of which are native to North China. Chipmunks were used only when squirrels were not available. In both of these animals intraperitoneal inoculation of blood direct from a Chinese patient suffering from louse-borne relapsing fever produced a non-febrile attack lasting usually 3 to 5 days, in which huge numbers of

TABLE I.

Typical Course of Relapsing Fever in a Splenectomized Squirrel (Sq. B, Chart 1).

March 25. Splenectomy performed.

March 29. Inoculated intraperitoneally with 0.5 cc. whole blood from a positive squirrel.

	Dates inclusive	Duration in days	Result of examination of tail blood				
			1st day	2nd day	3rd day	4th day	5th day
		days					
First attack	Mar. 30–Apr. 3	5	++	++	++++	+++	+++
First interval	Apr. 4–11	8	Daily examination negative for 8 days				
Second attack (1st relapse)	Apr. 12–14	3	+	+	+++		
Second interval	Apr. 15–20	6	Daily examination negative for 6 days				
Third attack (2nd relapse)	Apr. 21–25	5	+	+	++	++	+++

The number of plus signs indicates relative intensity of blood infection. + = one spirochete to ten or more fields. ++++ = 20 or more spirochetes to a single oil immersion field.

The tail blood was examined daily for 30 days after the third attack and was always negative.

spirochetes were present in the animal's blood. A second attack, however, never occurred, and the strain at first employed died out after the sixth transfer.

The observation of Tournade (12) that splenectomy increased the susceptibility of the wild rat to infection with *S. recurrentis* suggested that this procedure might produce relapses in squirrels and chipmunks,

[1] In a previous paper (13) this squirrel was erroneously called *Sciurus vulgarus*.

and this was found to be the case. The effect of splenectomy on the course of the disease has already been reported (13). Not only a second attack (first relapse) but a third attack (second relapse) occurred in many of the animals. Table I shows a typical course of infection in a splenectomized squirrel. Kritschewski and Rubinstein (14) have recently shown that splenectomy markedly increases the mortality of infection with *S. recurrentis* in white mice.

In the present experiments the original strain of spirochetes was transferred from squirrel to squirrel at the height of the first attack. When relapses occurred with this strain the relapse spirochetes were likewise transferred to separate series of squirrels. These squirrels,

TABLE II.

Duration of Existence of Strains Maintained by Transfer.

Strain No.	Duration	No. of transfers
	days	
I	132	38
II	106	29
III	98	26
IV*	75	20
IV†	32	8
Va††	54	16
Va§	39	10
Vb	50	14
VI	22	6

* Isolated from Sq. C, Chart 1; † isolated from Sq. D, Chart 1; †† isolated from Sq. F, Chart 1; § isolated from Sq. E, Chart 1.

in turn, usually had relapses, and when this occurred the spirochetes appearing in the relapses were tested against the monovalent immune sera produced by previously isolated strains. Whenever a strain of spirochetes was encountered which could not be agglutinated by any of these sera it was considered to be a new strain and was preserved by transfer into new squirrels.

By this method six strains of spirochetes were isolated, which retained their specific agglutinating characteristics during the period of our observations, extending from March 21, to July 30, 1927. Table II shows the duration of existence of the six strains. The history of these strains is given in detail below.

Technique.

Splenectomy.—This was performed under ether anesthesia through a small transverse incision below the left costal margin. The pedicle was ligated, and the wound closed with silk sutures and metal clips. A localized area of necrosis frequently developed about the tail of the pancreas where this had been accidentally ligated. Very few animals died as a direct result of the operation, and general peritonitis did not occur.

Transfer of Spirochetal Blood.—Infection of the first squirrels was accomplished by the intraperitoneal injection of venous blood from a human case of relapsing fever. For transfer from squirrel to squirrel, blood was taken from the heart or the saphenous vein. 0.5 cc. of blood from an animal with a moderately heavy infection usually produced demonstrable blood infection within 12 to 18 hours.

Examination of Blood for S. recurrentis.—The tail blood of all infected animals was examined daily by dark-ground illumination. No animal was declared negative without a search of at least 5 minutes.

Collection of Immune Sera.—Blood was taken from the saphenous vein or by heart puncture. The sera were pipetted off from the clot and stored in the refrigerator. Sera appeared to retain their original agglutinative titer for over 2 months, that is, until the present study was completed.

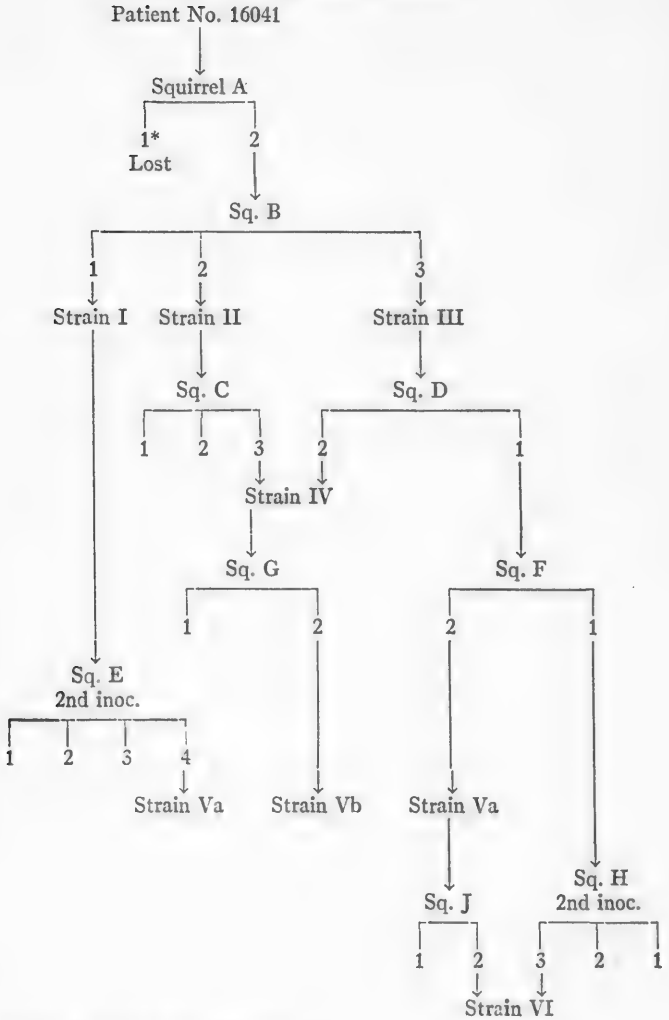
Agglutination Tests.—Preliminary tests suggested that a dilution of less than 1–50 sometimes produced non-specific agglutination. This dilution was the lowest used in differentiating strains of spirochetes, but in measuring the curve of agglutinins in single animals dilutions of 1–10 and 1–20 were also used. Sera were diluted with normal saline, and controls consisting of saline and negative sera were employed. For the spirochete suspension the tail blood of strongly positive squirrels was used. This was found to be more practical than the removal of blood into citrate solution and the subsequent removal of the red blood cells, because the latter procedure sometimes led to the rapid death or spontaneous agglutination of the spirochetes. With moderate or large numbers of spirochetes in the blood, the red blood cells did not interfere with the reading of the tests. Tests were performed with slide-cover slip preparations and observations were made at once by dark-ground illumination. A second observation after half an hour was found to be of no value. This technique is practically the same as that employed by Cunningham (6).

History of Strains Isolated.

The original strain of spirochetes used for these observations was obtained from a Chinese woman suffering from relapsing fever in her first attack of fever. 4 cc. of whole blood were injected intraperitoneally into a squirrel (Squirrel A, Chart 1). This squirrel's spleen had been removed 5 months previously. Spirochetes appeared in the

CHART 1.

Lineage of Strains Isolated.



*Arabic numerals refer to attacks.

tail blood the day following inoculation and the attack lasted 5 days. It happened that no new squirrels were available at this time, so that the spirochetes of this attack were lost. After a negative interval of 7 days, spirochetes again appeared in the tail blood. This second-attack (first-relapse) strain of spirochetes was thereafter maintained in squirrels by transfer during their first attack, and became Strain I in the following experiments.

Chart 1 shows the lineage of the strains isolated. It has been reduced to the simplest possible form by omitting all animals except those from which new strains were isolated. It will be seen that Strains II and III originated from the second and third attacks, respectively, of a squirrel (Sq. B) inoculated with Strain I; that Strain IV was obtained from two sources, namely from the third attack of a squirrel (Sq. C) inoculated with Strain II and from the second attack of one (Sq. D) inoculated with Strain III. Strain V is divided into two closely related substrains, Va and Vb. Strain Va was obtained both from a fourth attack[2] in a squirrel (Sq. E) inoculated with Strain I and from a second attack in one (Sq. F) inoculated with Strain III. Strain Vb appeared in the second attack of a squirrel (Sq. G) inoculated with Strain IV. At first all of the Strain V spirochetes appeared to be identical, since they were each agglutinated by the monovalent serum produced by the other. It was found, however, that the spirochetes designated as Strain Vb had a definite relationship with Strain III, while those designated as Strain Va failed to show this relationship but did show a relationship to Strain II (see later section on Relationship of Strains). Finally Strain VI originated from the third attack of a squirrel (Sq. H) inoculated with Strain III and from the second attack of a squirrel (Sq. J) inoculated with Strain Va. It was necessary to close the present study soon after the isolation of Strain VI, because of inability to secure more animals.

Sequence of Strains.

A consideration of the sequence in which the different strains of spirochetes appeared in relapses is important. The identity of each relapse strain was determined either by testing the spirochetes which

[2] The last three of these attacks were produced by a second inoculation with Strain I.

appeared during an attack against monovalent immune sera, or by observing what additional agglutinins appeared in the animal's blood after the attack. In some cases both procedures were used. Tests of an animal's serum after an attack did not always indicate clearly what strain had been present during the attack, for agglutination was sometimes equally strong with the sera of two different strains. This is because of the relationship existing between certain strains, as will be shown later. Table III contains those cases in which there was a clear-cut indication of the sequence. It will be seen that in animals inoculated with Strain I, Strain II was the only strain which appeared in the second attack, and Strain III the only one which appeared in the third attack. When other strains were inoculated, however, the sequence was not so uniform. Strain II was usually followed in the

TABLE III.

Sequence of Strains in Relapses.

Strain inoculated	Number of cases in which each strain appeared in subsequent attacks (relapses)													
	Second attack strain							Third attack strain						
	I	II	III	IV	Va	Vb	VI	I	II	III	IV	Va	Vb	VI
I	0	9	0	0	0	0	0	0	0	4	0	0	0	0
II	1	0	7	0	0	0	0	0	0	0	2	1*		0
III	0	0	0	2	1	0	0	0	0	0	0	0	0	1
IV	0	1	2	0	0	3	0	0	0	0	0	0	0	1
Va	0	0	1	0	0	0	1							

* Immune serum of this squirrel agglutinated spirochetes of both Strains Va and Vb.

second attack by Strain III, but once there was a recurrence to Strain I, and in the third attack Strain IV appeared twice and Strain V once. Strain III was followed in the second attack by Strain IV or Va, showing similarity here to the third attack after inoculation with Strain II. Strain IV was followed in the second attack three times by Strain Vb, twice by Strain III and once by Strain II. Strain Va was followed once by Strain III and once by Strain VI. Strain VI appeared also in the third attack once after inoculation with Strain III and once after inoculation with Strain IV. In short, inoculations with Strain I gave a uniform sequence, while after inoculations with other strains

the sequence of strains was not uniform. In some cases reversion to earlier strains occurred, while in other cases new strains appeared.

Although the figures presented in Table III are small for most of the strains, they are sufficient to illustrate an important principle connected with the development of relapses. This principle is the relationship of strains, on the basis of which all except one of the sequences which are shown in Table III can be explained. This relationship between certain strains will now be considered.

Relationship of Strains.

Cunningham (6), in his experiments with squirrels in Madras, India, was able to isolate only two strains of spirochetes, and these strains alternated in their appearance. Whenever his Strain I was inoculated Strain II appeared in the relapse, and whenever Strain II was inoculated Strain I appeared in the relapse. In the present work,

TABLE IV.

Agglutinins in Serum of Squirrels after First Attack.

Strain inoculated	No. of sera tested	No of sera containing agglutinins in 1–50 dilution for strains			
		I	II	III	IV
I	10	10	0	0	0
II	11	0	11	0	0
III	5	3	0	5	0
IV	7	0	2	0	7

although the strains already described were definitely distinct, certain relationships were found to exist which correspond in principle with Cunningham's findings.

These relationships were demonstrated by the agglutination tests performed with sera taken from animals after the completion of one or more attacks. Table IV shows the results of agglutination tests performed with sera taken after the first attack of Strains I, II, III and IV. It will be noted that, in the case of serum from Strains I and II, agglutinins were demonstrated only for the homologous strain of spirochetes. In the case of Strain III, however, three out of five animals developed agglutinins not only for Strain III but also for

Strain I. Similarly in the case of Strain IV, two animals out of seven developed agglutinins for Strain II as well as for Strain IV. This indicates a relationship between Strains I and III and between Strains II and IV.

The relationship between Strains I and III is further demonstrated by studying the agglutinins developed after the second attack in squirrels inoculated with Strain II. The results of these tests are shown in Table V. It will be seen that nine of the ten sera tested still possessed the agglutinins for Strain II which they had acquired as a result of the first attack. As a result of the second attack, all ten of the sera contained agglutinins for Strain I and five of them possessed in addition agglutinins for Strain III. There was more uniformity of agglutinin formation for Strain I than for Strain III, although, as has been shown already in Table III, the Strain III

TABLE V.

Agglutinins in Serum of Strain II Squirrels after Second Attack.

No. of sera tested	No. of sera containing agglutinins in 1–50 dilution for strains			
	I	II	III	IV
10	10	9	5	0

spirochetes appeared more often than Strain I in the second attack of squirrels inoculated with Strain II.

Strain Va was found to be related to Strain II, by the fact that after a first attack with Strain Va the animal's serum in two cases out of three agglutinated both the Strain II and the Strain Va spirochetes.

A relationship was shown to exist between Strain III and Strain Vb, by the fact that the Strain III spirochetes were agglutinated by univalent Strain Vb serum in all of the seven cases where the test was performed. Strains III and Vb were not identical, however, since the Strain Vb spirochetes were never agglutinated by univalent Strain III serum.

Strain VI was isolated such a short time before the close of the study that its relationship to other strains could not be investigated by means of agglutination tests.

In recapitulation it may now be stated that relationships were shown by agglutination tests to exist between Strains I and III, between Strains II and IV, and II and Va, and between Strains III and Vb. If one refers again to Table III it is evident that these relationships are all between strains which occurred, or might theoretically occur, in alternate attacks. If we now apply this observation to Strain VI in an attempt to ascertain its relationship to other strains, it appears (Table III) that this strain was related both to Strain III and to Strain IV, since it was responsible for the third or alternate attack in animals inoculated with each of these strains. Strains which were found to be related to one another did not occur in consecutive attacks, except once, when Strain II spirochetes appeared in a first relapse of Strain IV.

Reinoculation Experiments in Non-Splenectomized Squirrels.

Twenty-four squirrels were inoculated without removing the spleen and had no relapse. After an interval of 10 to 20 days, each one was reinoculated with the strain with which it had been infected originally. All except one showed itself immune, the tail blood remaining negative after the reinoculation. Following this, other strains were inoculated in succession, with shorter intervals between inoculation. The inoculations were usually successful, producing a blood infection lasting from 1 to 5 days. No squirrel, however, became infected with more than four strains. Among the twenty-four squirrels in this series, the results were as follows: 6 squirrels became positive to 4 different strains, 11 squirrels became positive to 3 different strains, 6 squirrels became positive to 2 different strains, and 1 squirrel became positive to 1 strain only.

The successful inoculation of as many as four different strains in succession emphasizes the individuality of these strains. The reinoculations were made at such short intervals that loss of immunity cannot be held responsible for the successful reinfections, for it is usual for antibodies against a given strain to be demonstrable in an animal's blood for at least a month after an attack with that strain. The following protocol illustrates this type of experiment.

Protocol.—Squirrel 1. Spleen not removed. Inoculated with Strain I. Tail blood positive for 3 days. After 16 days negative the squirrel was reinoculated with Strain I. Tail blood negative for 4 days. Then it was inoculated with

Strain II. Tail blood positive for 2 days. After 9 days negative, it was reinoculated with Strain III. Tail blood positive for 2 days. After 5 days negative, it was reinoculated with Strain IV. Tail blood positive for 3 days. Total interval from second inoculation with Strain I to inoculation with Strain IV, 22 days.

The agglutinins and protective immune substances which were developed by a single attack seemed to be about equal in strength in normal and in splenectomized squirrels. Reinfection with a previously inoculated strain was successful only once in a normal and once in a splenectomized animal. Agglutinins to a titer of 1–12,800 frequently developed in the serum of both normal and splenectomized animals. The strength of the agglutinins seemed, in general, to be directly proportional to the intensity and duration of the attack. It may be stated that neither specific immunity nor agglutinin formation seemed to be influenced by the presence or absence of the spleen.

DISCUSSION.

A. Strain Relationships, Relapses and Cure.

The fact that the strains of spirochetes which appeared spontaneously in consecutive attacks in splenectomized squirrels did not (with one exception) appear to be serologically related to each other, while those which appeared in alternate attacks were so related, is explainable on the basis that, at the close of an attack, the spirochetes find themselves in an exceedingly unfavorable environment, in which only those can survive which possess, or can quickly develop, biological affinities most different from those of the original strain. These biologically different spirochetes produce the second attack, at the close of which they themselves encounter a very unfavorable environment. Again only those can survive which possess, or can quickly develop, biological affinities which are very different from the second strain. Since the unfavorable conditions which were present at the close of the first attack probably still exist at the end of the second attack, the original strain cannot again appear, but a third strain of spirochetes does appear, whose biological affinities are more like those of the original strain than like those of the first-relapse strain. Assuming that a few organisms survive in the brain and blood stream even after the last attack, a fact which Buschke and Kroo (9) and Kudicke, Feldt and

Collier (10) have established, it is probable that clinical cure occurs when the surviving spirochetes find their environment too unfavorable for further multiplication in any great number.

The question arises whether the spirochetes which survive after an attack already possess biological affinities different from the original strain before they meet the unfavorable conditions which cause the destruction of their fellows, or whether their biological affinities change as a result of the unfavorable environment. The evidence at hand seems to indicate that the change does not occur before the development of the unfavorable environment but is rather the result of it; for if spirochetes which were biologically different from the original strain had existed before the crisis, one would expect them to have multiplied parallel with the original strain and to have given rise to antibodies in demonstrable amount, which they did not do.

The number of attacks which will occur before the medium becomes too unfavorable for the further occurrence of relapses must depend upon the interplay of several biological factors. Among these the three following are important:

(*1*) *Differences between Types of Spirochetes.*—It is well known that in the tick-borne type of human relapsing fever, the attacks are more numerous, are of shorter duration and occur at shorter intervals than in the louse-borne disease. The shorter attacks are probably due to a biological difference in the parasites, while the shorter intervals and larger number of attacks may be due, in part at least, to a less intense stimulation of immune bodies in the host as a result of the shorter attacks.

(*2*) *Susceptibility of Host.*—Certain animals like the guinea pig are naturally refractory to infection with *S. recurrentis*. Others, like the species of squirrel used in these experiments, normally have one attack but no relapse. Man is the ideal host and usually has the largest number of relapses.

(*3*) *The spleen* apparently plays an important rôle in preventing a multiplication of the parasites in animals like the wild rat which are naturally refractive to infection (Tournade (12)). It also prevents relapses in moderately susceptible animals like the Chinese squirrel. The exact nature of this protection is at present unknown, but the above observations indicate that it is not of the same nature as the

specific immune bodies which are produced in response to the infection. Two facts support this conclusion. In the first place, non-splenectomized animals could usually be reinfected by the inoculation of a second strain of spirochetes, although a spontaneous relapse never occurred in them. This indicates that the spleen did not produce any wider range of immune bodies in normal animals than in splenectomized animals. It also indicates that the protective power of the spleen is relatively slight, since a second attack which would not occur spontaneously could be produced by massive reinoculation. In the second place, splenectomized animals developed as efficient an immunity and as strong agglutinins against the organisms which appeared in their blood as did non-splenectomized animals. This indicates that the protection afforded by the spleen is not due to its production of specific antibodies. Further light on the protective function of the spleen might be obtained by determining whether, at the close of the first attack in animals which do not have a relapse, the spirochetes are all killed; or whether a few survive as they do in animals which have relapses. If they are all killed, the spleen would seem to assist in their destruction, whereas if they persist over a considerable period of time, it would appear that the spleen merely has the power to inhibit their multiplication.

B. Zones of Antibody Formation.

It will be recalled that when Strain III, IV or V was inoculated into an animal, serum taken after the first attack often agglutinated not only the strain of spirochetes inoculated, but also a related strain. When Strain I or II was inoculated, however, serum taken after the first attack agglutinated only the strain inoculated. To understand this, one must remember that the designation of any strain of organisms as Strain I is entirely arbitrary, and that it simply means that one does not possess any preceding strain. Our Strain I was a first relapse spirochete from a squirrel which had been inoculated from a patient. We know that a change of strain must have taken place in the squirrel. Another change may have taken place in the louse which transmitted the disease to the patient, and it is possible, also, that the louse acquired its spirochetes from another human being during a relapse. If we had possessed all of these preceding strains of spiro-

chetes, it is reasonable to suppose that some of them could have been agglutinated by sera taken after the first attack with our Strain I or Strain II. There is no reason to suppose that Strains I and II were not capable of giving rise to as wide a range of antibodies as the other strains which were isolated in these experiments. This fact is emphasized because it is important in considering the serological reactions in any group of spirochetes, and has apparently been overlooked in much of the preceding work on relapsing fever.

There is another conception, however, which may explain why our Strain I spirochete was sometimes agglutinated by monovalent Strain III serum, whereas our Strain III spirochete was never agglutinated by monovalent Strain I serum. When Strain III originally developed in the third attack of an animal inoculated with Strain I, it developed in the presence of antibodies which would destroy any Strain I spirochetes which might appear. However, when these Strain III spirochetes were inoculated into a new animal which possessed no antibodies against Strain I, spirochetes of the closely related Strain I which might develop during the first attack would not be destroyed, but would multiply along with the Strain III spirochetes, and give rise, at the end of the attack, to Strain I antibodies in serum which was supposedly monovalent for Strain III. If this conception is correct, one must assume that, since Strain I spirochetes were agglutinated by some Strain III sera but not by others, the Strain I spirochetes developed in some animals inoculated with Strain III but not in others.

Kudicke and Feldt (5) found that a component of their Strain I (Ausgangsstamm) developed in their relapse strains when these were passed through normal mice, and that this component increased in subsequent passages until the relapse strains ultimately reverted completely to their Strain I. In the present study, no reversion to an older strain was observed. The development of agglutinins for older, closely related strains was the only approach to such a reversion. Each strain possessed the same agglutination reactions at the end of our study as it had after its first transfer to a new squirrel. This coincides with the observations of Cunningham (6), who also worked with squirrels. Toyoda (11), on the other hand, who worked with mice, found that his relapse strain reverted to the original strain after

eighteen passages. Whether the type of experimental animal had anything to do with this difference in results is a question which deserves further investigation.

It seems probable that each strain of spirochetes which appears during an attack of relapsing fever contains its own peculiar group of components, the exact range of which depends largely upon the range of the antibodies which were present in the environment in which the strain originated. When a number of relapse strains are isolated in experimental animals by artificial transfer, it is apparent that the components making up those strains may overlap to a considerable degree.

Fig. 1 has been constructed in order to illustrate the relationships of our strains and the gradual increase in the complexity of their sero-

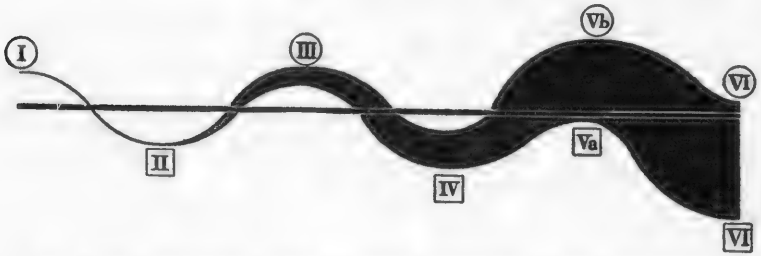


FIG. 1. The broadening zone of strain relationships in the descendants of Strain I.

logical relations. The wavy form of the curve represents the alternation in serological relationships between the spirochetes which appeared in consecutive attacks. The strains above the base line and enclosed in circles were related to each other, and those below the base line and enclosed in squares were likewise related. The curve is linear as far as Strain II because the sera produced by Strains I and II failed to agglutinate any other strains. The gradual broadening of the curve beginning just beyond Strain II, until it ultimately extends both above and below the base line at Strain V, represents the gradually broadening zone of relationship among the strains which were descendants of Strain I. The curve must be broader at III than at I because Strain III sometimes included a Strain I component, but was not identical

with Strain I. At V the curve must extend both above and below the base line, because Strain Vb was related to Strain III, while Strain Va was related to Strain II. At VI the curve must also extend both above and below the base line, because, since Strain VI appeared as the third-attack spirochete both in an animal inoculated with Strain III and in one inoculated with Strain IV, it was apparently related to both of these strains.

It is probable that a similar curve could have been constructed if the work had been begun either with an earlier strain than Strain I or with one of the later strains, say Strain V. In the former case the curve would show broader relationships in the region of the present Strain I, perhaps carrying it both above and below the base line. In the latter case, on the other hand, the curve would be linear for the distance from Strain V to Strain VI where it is now broadest.

There is evidence that a number of strains of *S. recurrentis* are present among human cases in an endemic region like North China. While the present work was in progress two other human cases yielded strains of spirochetes which could not be agglutinated by any of our sera. The components making up these strains evidently did not include any of those which made up our strains. Toyoda (7) also found that among ten cases of relapsing fever in a Manchurian mine there were at least three different strains of spirochetes. How many different immunological strains of *S. recurrentis* there may be in China, or in the entire territory where the louse-borne relapsing fever is found, could be determined only by the collection and testing of organisms from a very large number of cases from various regions.

The serological studies of many previous workers, as well as the present experiments, have demonstrated that agglutination and immunity tests are not satisfactory criteria for differentiating the spirochetes of relapsing fever from various parts of the world. Until some better criterion for their differentiation appears, it is reasonable to consider that all the spirochetes of relapsing fever belong to one species, *S. recurrentis*. Within this species there are two biological varieties, the louse-borne parasite, which everywhere produces relatively long attacks separated by long intervals in human beings, and which is difficult to transfer to white rats and mice; and the tick-borne parasite, which produces short attacks separated by short intervals in human

beings, and which is easily transferred to white rats and mice. The erection of several species, because of geographical or immunological differences, or differences between vectors, seems to be unwarranted.

SUMMARY AND CONCLUSIONS.

1. Squirrels and chipmunks were found to be susceptible to infection with the *Spironema recurrentis* of North China, but no relapses occurred in normal animals.

2. Splenectomy caused an increase in the intensity of the infection and the appearance of one or two relapses.

3. By inoculating splenectomized squirrels with a single human strain of spirochetes, six different strains were produced as a result of relapses.

4. The strains all retained their specific agglutinating characteristics during the period of observation. The oldest strain was observed for 132 days and through 38 transfers, the strain last isolated for 22 days and through 6 transfers.

5. The sequence of strains in relapses was not always the same. Sometimes new strains were produced, at other times there was reversion to an older strain.

6. Certain relationships between strains were established. The related strains were always those which appeared, or theoretically might have appeared, in alternate attacks. In only one case did related strains appear in two consecutive attacks.

7. Squirrels whose spleens were not removed had only one attack and developed immune substances only against the strain inoculated or against closely related strains. It was possible by reinoculation to produce infection in such squirrels with as many as four different strains in succession.

8. The spleen has a protective influence against the development of the relapse in the squirrel, but apparently does not control the formation of specific immune substances.

9. An explanation of the clinical course of relapsing fever is suggested on the basis of the observations recorded.

10. There is, at present, no justification for the division of the spirochetes of relapsing fever into different species.

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FACTORS INVOLVED IN THE PRODUCTION OF IMMUNITY WITH PNEUMOCOCCUS VACCINE.

I. Active and Passive Immunity during the First Seven Days after Injection of Antigen.

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Inasmuch as pneumonia is a self-limited disease ending by crisis or lysis generally between the 7th and 10th days, 3 to 5 days are frequently afforded in which an attempt may be made to produce active immunity before the natural termination of the disease takes place. At the suggestion of Dr. A. R. Dochez, a study was therefore undertaken of the onset and rate of development of pneumococcus immunity. As a result of recent work in oxygen therapy, it appeared to the author (1) that the life of the pneumonia patient was at times prolonged by the inhalation of 40 to 50 per cent oxygen, a circumstance that would further increase the possibilities of pneumococcus vaccine in an individual case. Any procedure that would initiate an earlier activity of the immunity mechanism seems therefore to have therapeutic possibilities. We wish to report preliminary animal experiments that have a bearing on this problem.

Historical.

In reviewing the subject, we shall present only such work as is concerned with the demonstration of specific immunization to the pneumococcus, and shall therefore omit the clinical reports of pneumococcus vaccine in the treatment of pneumonia as being apart from the present purpose. The development of immunity after introduction of pneumococcus organism has been shown by a number of workers. A great variety of methods of preparation of the organism has been used, employing the intact cell and extracts or solutions of the cell. The onset of demonstrable immunity in the studies of different investigators has been generally

between the 5th and 14th day after injection. Deviations from these results will be taken up in the individual instances. As we are concerned particularly with the attempt to secure a vaccine that would be applicable to the treatment of pneumonia, we shall stress chiefly the development of immunity during the first 7 days after injection.

The first demonstration that the serum of animals injected with pneumococcus possessed immune properties was made in 1891 by Foa and Carbone (2), Emmerich and Fowitzky (3), and G. and F. Klemperer (4). Neufeld (5) (1902) employed the bacteria separated from broth by centrifugalization, believing that the substances formed in the medium during growth were harmful. Wadsworth (6) (1912) found that the immunity produced by whole cultures was greater than that obtained by the injection of the bacteria alone. He also employed filtrates from the culture material after precipitation with ammonium sulfate. In both instances active immunity was produced in the rabbit 8 to 13 days after the injection. In 1913 Dochez and Gillespie (7) published their classification of pneumococci which made possible more specific study of individual strains. Lister (8), employing a similar classification in South Africa, made an intensive study of prophylactic inoculation against pneumococcus infection in the rabbit and in man. He found the factor of dosage was of considerable importance; doses limited to hundreds of millions of pneumococci were of little value, whereas 1 to 10 billion organisms administered intravenously both in the rabbit and in man were effective in immunizing the subject.

After three intravenous injections at weekly intervals, the rabbit was able to withstand a lethal dose of virulent pneumococcus culture. 4 to 5 days after the first injection, the serum of the animal in a few instances showed evidence of agglutinins when 3 volumes of serum were used to 1 of culture. One African native who received an intravenous inoculation of 10 billion the first dose, 20 billion the second dose, and 40 billion the third dose, showed agglutinins (with 3 volumes of serum) 6 days subsequent to the first injection. The vaccine was prepared from 18 hour blood agar cultures.

When the vaccine was administered subcutaneously, using from 2 to 12 billion organisms, no agglutinins were demonstrated even after three injections. When 20 to 30 billion doses were administered, agglutinins appeared 7 days after the second injection. In a later article, Lister (9) took his culture from human blood agar slants, inoculating it into trypsinated bullock heart broth for 8 hours, and then transferring this culture into similarly prepared broth for an incubation period of 6 to 7 hours. The cocci were finally suspended in saline, heated to 60° for 1 hour, and phenol added to a concentration of 0.5 per cent. Other preparations of vaccine such as (1) the filtrate after passing broth cultures through Berkefeld filters, (2) the cocci separated from broth cultures by electrolytic methods, (3) the pneumococci from 40 hours incubation in glucose broth cultures he found relatively inert as immunizing agents.

Still other types of vaccine have been employed: sensitized bacteria (Levy and

Aoki (10)), bacteria subjected to freezing (Cole (11)), bile extracts of pneumococcus (Neufeld (12), Vetrano (13), Cole (11)), glycerol extracts (G. and F. Klemperer (4)), and cultures precipitated by alcohol and dissolved in water (Wadsworth (6)). In all these instances, the immunity was slight, according to Cole (11), and never as good as from the injection of heat-killed bacteria. He found that better immunity was established in the rabbit by small daily doses than by larger doses at weeky intervals, agglutination (++ with equal parts of serum and culture) appearing on the 11th day after the first injection. The serum also protected a mouse against 0.001 cc. of virulent pneumococcus culture.

Wadsworth (14) recently recorded quantitatively the degree of immunity which he obtained with a standardized vaccine made of meat infusion broth with 0.5 per cent glucose. After three subcutaneous injections of Type II vaccine at weekly intervals, there was slight active immunity after the last injection. Thus, of three mice injected with 0.00001 cc. culture, one died, and of three injected with 0.0001 cc. culture, two died.

Larson and Nelson (15) reported that the addition of sodium ricinoleate to a virulent culture of pneumococcus so that the final dilution of the soap is 0.1 per cent caused the organisms to lose their pathogenicity. When 10 cc. of such a culture were injected intravenously into a rabbit, large amounts of agglutinins were present in the blood stream 24 hours after. Cecil (16) has produced active immunity in monkeys after three subcutaneous injections of Pneumococcus Types I and II and to a less extent in Type III. After a single large subcutaneous injection in man of 8 billion each of the three types, agglutination (+ in 1–1 dilution) was observed in some instances 7 days after. The vaccine was made by growing the organisms in 0.5 per cent glucose broth for 12 to 14 hours. Mackenzie (17) found that an intraperitoneal injection of 0.25 cc. of a heat-killed 18 hour culture produced active and passive immunity in guinea pigs 6 days after the injection although no agglutinins were demonstrable. Perlzweig (18) noted active immunity 10 days after a single subcutaneous injection of Pneumococcus Type I in mice. He also immunized mice actively with the protein fraction obtained by treating pneumococci with anhydrous sodium sulfate and by solution of pneumococci in bile salts and precipitation with alcohol. Ferry and Fisher (19) found that the centrifugate from broth cultures or saline suspensions of pneumococcus were more effective as an immunizing agent than the sedimented bacteria. White mice who were given two doses of 0.5 cc. of these extracts subcutaneously at weekly intervals were immune against 0.001 cc. of culture 1 week after the last injection. Wright (20) found that immunized rabbits cleared the blood of injected virulent pneumococci more effectively than normal rabbits. This increased resistance to septicemia was observed as early as the 3rd day following a single intravenous injection of heat-killed bacteria. Armstrong (21) has reported passive immunity in rabbits and active immunity in mice both beginning on the 4th day (occasionally on the 3rd day) and increasing to the 6th day after injection of killed pneumococcus vaccine.

In 1917 Dochez and Avery (22) pointed out that the pneumococcus elaborates a soluble specific substance which is found in the broth culture of the organism and frequently in the body fluids of the host as well. Recently, Avery and Heidelberger (23) have separated the constituents of the pneumococcus cell into two chemically and immunologically distinct substances, one protein and the other carbohydrate. The protein fraction is less specific than the intact bacterial cell, creating antibodies for the protein common to all groups of pneumococci when injected into animals. The carbohydrate, which has been shown to be of polysaccharide nature, is highly and specifically reactive only with the antibacterial serum of the same type of pneumococcus as that from which the substance is derived. However, when dissociated from combination with other cell elements, it is incapable of inducing antibody formation. They state that solutions and extracts of pneumococci behave as solutions of pneumococcus protein, *i.e.*, produce antibodies reactive only with the protein. Sera prepared from filtered solutions of disintegrated cells free from formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria. Schiemann and Casper (24) very recently reported that a specific soluble substance which they made employing a technique similar to Avery and Heidelberger was antigenic.

The literature recited above presents considerable divergence of opinion in regard to various problems in pneumococcus immunity. Our experiments have mainly emphasized two factors: (1) the onset and rate of development of pneumococcus immunity, (2) the character of immunity produced by the use of the intact organism as compared with that produced by a solution or extract from the bacterial cell.

Methods.

Numerous experiments have been conducted in the effort to prepare an antigen which would be particularly effective in producing early immunity. The detail of these findings will be considered at a later date. A fact originally mentioned by Neufeldt was suggested by our experience, namely, that the highly virulent organism provokes a more marked immunity than an organism of low virulence. We have attempted to prepare our antigen from a pneumococcus culture that was fatal to a mouse in 10–7 dilution, and preferably passed through a mouse immediately before using.

Human serum was employed in a concentration of 2.5 per cent in beef infusion broth in order to preserve virulence to the last stage and in order to augment

growth. The addition of glucose for stimulating growth did not appear to be satisfactory because of the readiness with which the organism was altered by growth in the culture medium. Young cultures were employed also with the idea of preventing autolysis. The incubation period was varied between 6 and 11 hours without appreciably influencing this factor.

Four different antigens were prepared in the following manner: A virulent Pneumococcus Type II (or Type I) culture was passed through a mouse in a preparation of the vaccine. 0.2 cc. of the heart's blood obtained from the animal immediately after death was inoculated into a test-tube containing 5 cc. of beef infusion broth with 2.5 per cent of human serum. After 8 hours incubation, this was used to inoculate flasks containing 250 cc. of similarly prepared broth, in the proportion of 0.1 cc. inoculum to 5 cc. broth. Incubation was carried on for 8 to 11 hours as was desired. The culture was then centrifuged, the supernatant broth poured off and saved. Care was taken not to loosen the sedimented bacteria at the bottom of the centrifuge tube which was now carefully rinsed with distilled water to wash off all broth adhering to it. Distilled water was finally added to dilute the bacteria to a concentration of 1 billion organisms to 1 cc. of water. (A nephelometer was used and checked by the Wright counting method.) The vaccine was sterilized by exposure to 60° for 1 hour. After it had cooled, tricresol was added to a concentration of 0.3 per cent. This is called in the text the 8 or the 11 hour serum vaccine.

The supernatant broth saved from the original culture was passed through a Berkefeld candle, bottled, and placed in the ice box. The preservative was added after culture had demonstrated no organisms had passed through the filter. This is called the broth filtrate.

A second lot of serum vaccine before sterilizing is shaken by hand for 5 minutes and centrifuged. The supernatant fluid is passed through a Berkefeld filter, bottled, and placed in the ice box. If sterile on culture, the preservative is added. This is called the filtrate of the shaken bacteria. The sediment remaining from the above is taken up in distilled water, heated for 1 hour at 60°, and allowed to cool before the addition of tricresol. This is called the washed bacteria vaccine.

Experiments.

Experiment 1.—A series of white mice were injected intraperitoneally with three different antigens derived from Pneumococcus Type II: (1) 11 hour serum vaccine, (2) Berkefeld filtrate of shaken bacteria, (3) washed bacteria vaccine. Each antigen was injected into five mice daily for 5 days. The dose of the serum vaccine and the washed bacteria vaccine was 0.2 cc., representing 200 million organisms. The dose of the filtrate was 0.4 cc., derived from 400 million organisms. At the end of the 5th day, all mice received an intraperitoneal injection of Type II culture in varying dilutions, as shown in Table I. The test culture was fatal to a mouse in 10–7 dilution.

TABLE I.

Active Immunity in Mice after Intraperitoneal Injection of (1) 11 Hour Serum Vaccine, (2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria Vaccine.

Antigen used and dose	Day after vaccination	Survival after Pneumococcus II injection of culture			Remarks
		.0001	.00001	.000001	
Vaccine 0.2 cc.	1st	24	28	30	All injections were administered intraperitoneally; antigen and test culture
		24			
	2nd	36	36	36	
			36	S	
	3rd	42	S	S	The surviving mice were subsequently injected with virulent Type I culture in corresponding doses. All died (within the same period that control mice died)
			42	S	
	4th	60	S	60	
			S	S	
	5th	S	S	68	
			S	S	
Filtrate of bacteria 0.4 cc.	1st	24	28	S	All antigens were prepared from an 11 hr. Pneumococcus Type II culture
			28	30	
	2nd		40	40	
			40		
	3rd	42	40	40	
			40	43	
	4th	S	S	S	
			S	S	
	5th		S	S	
		S	S	68	
Washed bacteria 0.2 cc.	1st	24	28	30	
			28	S	
	2nd	36	40	40	
			40	40	
	3rd	42	40	40	
			40	43	
	4th	S	62	62	
			S	S	
	5th	S	S	S	

Controls	.01	.001	.0001	.00001	.000001	.0000001
	15	15	25	35	68	85

As noted in Table I no protection was evidenced in any of the vaccinated animals on the 1st and 2nd days. Immunity began on the 3rd day in the case of the animals who received the serum vaccine and on the 4th day in those who received the washed bacteria vaccine and the filtrate of the shaken bacteria. The immunity was further increased on the 5th day. The mice who survived from this experiment were injected with a virulent Type I culture in corresponding doses to the Type II culture. All died.

Active immunity is therefore demonstrated on the 3rd day after injection of the serum vaccine, and on the 4th day in the case of the

TABLE II.

Active Immunity in Mice after Injection of 8 Hour Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 400 million organisms	1st	40	40	60	60	Vaccine and test culture administered intraperitoneally
	2nd	60	40	40	24	
	3rd		S	S	90	
	4th		S	S	S	
	5th		S	S	S	
	6th	40	S	S	21	
	7th	S	40	S	S	
Controls		40	21	40	96	

washed bacteria vaccine and the filtrate of the shaken bacteria. It is progressive in degree and specific for type. The failure of any of the vaccinated animals to survive an injection of Type I culture indicates that the protection against Type II culture was not due to increase in general resistance but rather to type-specific immunity. This was produced both by the use of the intact cell and by the water-soluble specific substance shaken off the bacteria.

Experiment 2.—(Table II.) An 8 hour serum vaccine was injected daily for 7 days into a series of white mice, four mice to each day. The dose was 0.4 cc., representing 400 million organisms, administered intraperitoneally. The test culture was of moderate virulence, 10–6 cc. being fatal to a mouse.

As shown in Table II, immunity is present from the 3rd to the 7th days inclusive.

Experiment 3.—(Table III.) An 8 hour serum vaccine was injected daily for 5 days into a series of white mice, five mice to each day. The dose was 0.2 cc.,

TABLE III.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of Low Virulence.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture					Remarks
		.01	.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 100 million organisms	2nd	S	S	S	S	S	Pneumococcus culture of relatively low virulence, 10–6 surviving and 10–5 fatal in 130 hrs.
	3rd	S	S	S	S	S	
	4th	S	S	S	S	S	
	5th	S	S	S	S	S	
Controls.....		36	22	36	130	S	

TABLE IV.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of High Virulence.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 200 million organisms	1st	14	44	44	44	Pneumococcus culture at maximum virulence, fatal in 10–7 cc.
	2nd	20	20	20	44	
	3rd	44	44	20	44	
	4th	20	53	S	S	
	5th	53	S	S	20	
Controls.....		14	14	20	20	

representing 200 million organisms. The test culture was of low virulence, 10–5 cc. being fatal in 130 hours. Immunity was demonstrated from the 2nd to the 5th day inclusive.

Experiment 4.—(Table IV.) A similar experiment to the above was performed, employing however, a highly virulent test culture, 10–7 cc. being fatal to a mouse. Immunity was obtained on the 4th and 5th days only.

TABLE V.

Passive Immunity in Rabbits after Intravenous Injection of (1) Serum Vaccine, (2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria Vaccine, (4) Berkefeld Filtrate of Broth Culture.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	.001	.0001	.000001	
Rabbit 4 pneumococcus II 11 hr. serum vaccine 5 billion organisms	3rd	S	60	S	18	45	45	Mice employed in Pneumococcus I infections were used previously in Pneumococcus II experiments in which they survived injections of virulent Pneumococcus II culture All rabbit injections given intravenously
			S	S		45	45	
	5th	S	S	S	45	45		
			S	S				
	7th	S	S	S				
			S	S				
	9th	S	42	S				
			42	S				
	15th	S	S	S				
			S	S				
	20th	42	S	S				
			S	S				
Rabbit 7 pneumococcus II filtrate of shaken bacteria 8 cc.	3rd	42	42	42				Concentration of antigens—1 billion organisms to 1 cc. distilled water
			42	60				
	4th	42	42	42				
			60	48				
	5th	S	S	S	42	45	45	
			S	S		45	45	
	6th	S	S	S	45	45	45	
			S	S		45	45	
	8th	S	S	S				
			S	S				
	11th	S	S	S				
			S	42				
Rabbit 5 pneumococcus II washed bacteria vaccine 5 billion organisms	3rd	S	42	S	45	45	45	
			S	S		45	45	
	5th	S	S	S	45	45	45	
			S	S		45	45	
	7th	S	S	S				
			S	130				
	9th	S	S	S				
			S	S				

TABLE V—*Concluded.*

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.100.	.0000.	.1000000.	.100.	.0000.	.1000000.	
Rabbit 6 Pneumococcus II filtrate of broth culture 10 cc.	3rd	36	S	60				
			S	60				
	4th	36	42	36				
			60	S				
	5th	S	S	S				
			S	S				
	7th	S	S	S				
			S	S				
	15th	S	S	S				
			S	S				
	20th	S	S	S				
			110	S				
Rabbit serum	Before	42	42	42				
Controls Pneumococcus II			42	42				
Controls Pneumococcus II		24	24	24				
Controls Pneumococcus I		48	48	60				

The foregoing experiments indicate that the onset of active immunity alters with the virulence of the test culture, appearing on the 2nd day after vaccination when an organism of low virulence is used, on the 4th day with an organism of very high virulence, and on the 3rd day with an organism of moderately high virulence. Varying the virulence of the organism represents the progressive increase in active immunity from the 2nd to the 5th day better than varying the number of organisms injected, for a highly virulent culture may cause death of all the animals even in high dilutions and a culture of low virulence may result in complete survival. This is in accordance with the results of Neill and Gaspari (28) who found that within a certain range (10–6 to 10–3 cc. of culture) the number of invading bacteria was without influence in determining the occurrence of infection and fatality.

Experiment 5.—(Table V.) Four antigens were used in this experiment: (1) 11 hour serum vaccine, (2) filtrate of shaken bacteria, (3) washed bacteria vaccine, (4) filtrate of broth culture. The dose of the serum vaccine and the washed bacteria vaccine was 5 billion organisms, the filtrate of the broth and the shaken bacteria was in each case derived from 10 billion organisms. Each antigen was administered intravenously to a rabbit, and blood taken, before and from the 3rd to the 9th day or longer. The mouse protection test was carried out according to the technique of Dochez (25). Thus, 0.2 cc. of rabbit serum was mixed with

TABLE VI.

Passive Immunity in Rabbits after Injection of (1) Serum Vaccine and (2) Berkefeld Filtrate of Shaken Bacteria.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.01	.001	.0001	.00001	
Rabbit 3 Pneumococcus II serum vaccine 2 billion organisms	1st	17	64	64	64	All antigens administered intravenously. Vaccine and filtrate made from 8 hr. serum broth culture
	2nd	30	30	30	64	
	3rd	30	S	S	S	
	4th	S	S	S	S	
	5th	50	30	S	S	
	6th	17	S	S	S	
	7th	S	S	S	S	
Rabbit 1 Pneumococcus II serum vaccine 2 billion organisms	3rd	64	64	S	S	
	4th	30	S	S	S	
	5th	46	S	S	S	
Rabbit 2 Pneumococcus II filtrate of shaken bacteria 2 cc.	4th	72	S	S	S	
	5th	S	S	S	S	
Controls.....		30	22	36	130	

varying dilutions of culture and injected intraperitoneally into mice. Survival in these as in the previous active immunity experiments is recorded when the mouse lives 6 days after injection. The blood drawn on the 3rd, 5th, and 6th days was also mixed with varying dilutions of Type I culture to determine whether the early immunity secured was a general non-specific increase in natural resistance or was entirely due to the development of Pneumococcus Type II specific protective substance. The test culture was of moderately high virulence, 10–6 cc. killing control animals as well as animals who received the serum of normal rabbits (*i.e.*, before their vaccination with pneumococcus antigen.)

As Table V indicates, the onset of passive immunity was on the 3rd day in the case of the serum vaccine and the washed bacteria vaccine. The broth filtrate and the filtrate of the shaken bacteria show a definite immunity on the 5th day. The immunity persists as long as observations in the experiments were conducted, from the 11th to the 20th day after injection. In a similar experiment noted in Table VI, the serum vaccine initiates passive protection on the 3rd day also, but the filtrate of the shaken bacteria produces an immune response on the 4th day. The rabbit sera of the animals whose blood protected against Type II culture failed to show any protection

TABLE VII.

Passive Immunity in Rabbit after Intravenous Injection of Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.0001	.00001	.000001	
Rabbit 1 Pneumococcus II 8 hr. serum vaccine 2 billion organisms	2nd	19	19	48	48	Virulence of pneumococcus culture was at its maximum, fatal in 10–7 cc.
	3rd	19	22	48	19	
	4th	19	48	48	49	
	5th	48	48	48	22	Mouse with immune horse serum was not protected against 10–6 culture.
	6th	19	48	72	72	
Immune serum		S	S	19	S	
Controls.....		22	48	48	72	

against Type I culture, demonstrating that the passive immunity produced was specific for type and not dependent upon increase of general resistance or on the common protein antibody.

Experiment 7.—(Table VII.) An 8 hour serum vaccine was injected intravenously to a rabbit, and blood drawn from the 2nd to the 6th day inclusive. The dose was 2 billion organisms. The test culture was at maximum virulence, 10–7 cc. being regularly fatal.

No animals survived in this experiment. The blood of rabbits varies to an extent in its power to elaborate protective substance, particularly in the case of a culture of maximum virulence.

Experiment 8.—(Table VIII.) A series of mice were inoculated intraperitoneally with an 11 hour (1) serum vaccine Type I pneumococcus and (2) filtrate of the shaken bacteria. The dose of the vaccine was 0.2 cc., representing 200 million organisms, and of the filtrate 0.4 cc., representing 400 million organisms. The test culture was of very high virulence, 10–7 cc. being fatal to a mouse.

Active immunity began on the 4th day in the animals vaccinated with the serum vaccine, and on the 5th day with the animals vaccinated with the filtrate of the shaken bacteria. This indicates again that the use of a test culture of very high virulence records the onset

TABLE VIII.

Active Immunity Following Intraperitoneal Injection of Type I Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	
Pneumococcus I 11 hr. serum vaccine 400 million organisms	1st	20	20	24	Vaccine and test culture administered intraperitoneally
			20	36	
	2nd	20	20	24	
		36	36	36	
	3rd	24	36	36	
			36	36	
	4th	36	S	S	
			S	48	
	5th	S	S	S	
			S	48	
Controls		20	24	36	
			20	24	

of immunity at a later date than one of lesser virulence, and thus registers the progressive increase in antibody response from the 3rd to the 5th day.

Experiment 9.—(Table IX.) In this experiment two antigens from Pneumococcus Type I were employed, (1) the Berkefeld filtrate of the shaken bacteria and (2) the filtrate of the broth culture. A comparison was made between the standard dose and a very large dose. A series of mice were inoculated intraperitoneally with 0.4 cc. of broth filtrate, a second series with 1.0 cc. of broth filtrate, a third series with 0.2 cc. of the filtrate from the shaken bacteria, and a fourth with 1.0 cc. of the filtrate of the shaken bacteria. Each antigen was injected daily into eight mice, and on the 7th day after the first injection, the test culture was administered to the

entire series, comprising mice injected 2 to 7 days after inoculation with antigen. The test culture was of high virulence, 10–7 being fatal to a mouse in 60 hours.

As will be seen in Table IX, all the mice injected on the 2nd day after vaccination died, 32 in number. Survival began on the 3rd day after injection in each series but usually in only one out of eight animals. In the case of the filtrate of the shaken bacteria, definite protection was present on the 4th day, increasing to its height on the

TABLE IX.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intraperitoneal Injection of (1) Berkefeld Filtrate of Shaken Bacteria, (2) Berkefeld Filtrate of Broth Culture.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Broth filtrate 0.4 cc.	2nd	40	40	40	58			All injections administered intraperitoneally. Filtrates made from 11 hr. serum vaccine, Pneumococcus Type I.
		40	58	58	58			
	3rd	58	58	58	40			
		58	58	58	S			
	4th	18	40	58	58			
		58	58	58	S			
	5th	40	40	58	40			
		58	58	S	S			
	6th	40	40	58				
		40	80	S	S			
	7th	40	S	58	S			
		40	S	S	S			
Broth filtrate 1.0 cc.	2nd	40	40	40				
		40	58	58	58			
	3rd	40	40	40	40			
		40	40	S	58			
	4th	40	40	58	S			
		58	S	58	S			
	5th	40	40	40	S			
		58	40	58	80			
	6th	40	48	58	S			
		40	58					
	7th	40	58	S	S			
		40	S	S	S			

TABLE IX—*Concluded.*

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Bacterial filtrate 0.2 cc.	2nd	18	40	40	58			
		40	40	40	58			
	3rd	40	40	40	40			
		40	S	40				
	4th	40	40	40	S			
		40	40	S	S			
	5th	18	S	S	S			
		40	80	S	S			
	6th	40	58	80	S			
		S						
	7th	40	S	S	18			
		40	S		18			
Bacterial filtrate 1.0 cc.	2nd	40	40	40	58			
		40	40	58	58			
	3rd	40	40		S			
		40	40	40				
	4th	40	40					
		40	80	S	S			
	5th	40	18	S	S			
		40	S	S	S			
	6th	40	40	S	S			
		58	58					
	7th	40	58	40	S			
		40	58	58				
Controls		40	40	58	58	58	60	
		40	40		58			

5th day, and remaining approximately stationary to the 7th. In fact, in this experiment there was a slight falling off in survival from the 5th to the 7th day. The increased dose (1.0 cc. of filtrate) gave slightly inferior results than the standard dose (0.2 cc.).

The immunity resulting from the standard broth dose (0.4 cc.) was less marked than that from the filtrate of the shaken bacteria. Animals survived from the 3rd to the 6th day in small numbers and on the 7th day, five out of eight survived. The large dose of broth (1.0 cc.) gave a less marked immunity response but here too, five out of

TABLE X.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intraperitoneal Injection of Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
Serum vaccine dose 0.2 cc.	2nd	44	44	70	All injections administered intraperitoneally
		44	44	70	
		44	44	44	Vaccine grown for 8 hrs. in 2.5 per cent serum broth
	3rd	92	92	SS	
		SS	SS	S	Numbers in table refer to length of time in hrs. which mice lived after injection. S indicates survival for 6 days or more
	4th	SS	SS	SS	
		S	S	S	
	5th	SS	SS	SS	
		S	S	S	
Dose 0.02 cc.	2nd	44	44	44	
		44	44	44	
			44		
	3rd	44	44	44	
		44	44		
		S	S	SS	
	4th	SS	92	SS	
		S	SS	S	
	5th	92	SS	SS	
		SS	S	S	
Dose 0.002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	SS	44	SS	
			92		
		S	S	S	
	4th	SS	SS	SS	
		S	S	S	
	5th	SS	SS	SS	
		S	44	S	
Dose 0.0002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	44	SS	44	
		44		44	
		S	S	S	
	4th	SS	44	92	
		S	SS	SS	
	5th	44	70	92	
		92			
		S	SS	SS	

Normal controls.....	.001	.0001	.00001	.000001	.0000001	.00000001	.000000001
	44	44	44	44	44	44	44

eight survived on the 7th day. It is observed that in only one instance (1 of 192 mice), did an animal resist 0.01 cc. of test culture. On the 5th day, however, the filtrate of the shaken bacteria produced an immunity sufficient to withstand an injection of 0.001 cc. of a culture with a virulence of 10^{-7}.

Experiment 10.—A series of mice were injected intraperitoneally with the following doses of Type I vaccine; (1) 0.2 cc., (2) 0.02 cc., (3) 0.002 cc., (4) 0.0002 cc.

TABLE XI.

Active Immunity after Intraperitoneal Injection of Pneumococcus Type I Culture to Which Sodium Ricinoleate Had Been Added.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
0.1 per cent sodium ricinoleate Pneumococcus Type I culture	1st	44	20	44	11 hr. Pneumococcus Type I broth culture exposed to a final concentration of 0.1 per cent sodium ricinoleate before injection
		44	44	44	
	2nd	44	44	44	
		44	44	44	
	3rd	44	20	44	
		44	44	84	
	4th	44	44	S	
		S	44	S	
Dose 0.4 cc.	6th	S	S	S	
		S	S	S	
	7th		44	44	
		S	S	S	
Dose 0.04 cc.	1st	20	20	20	
		20	20	20	
	4th	44	44	S	
		64	64	S	

Normal controls.....	.001	.0001	.00001	.000001	.0000001	.00000001
	44	44	44	44	44	44

1 cc. of the vaccine contained 5 billion organisms. Twelve mice were inoculated daily with each antigen, and on the 5th day a test culture of Pneumococcus Type I was injected intraperitoneally into the entire group. The virulence of the culture was such that 10^{-9} cc. was fatal to a control animal.

As seen in Table X all the 2nd day mice (48 in number) as well as all the control animals died. Survival began on the 3rd day and was

more complete on the 4th and 5th days. The immunity produced was fairly constant in the range of dosage between 0.2 cc. and 0.002 cc., but began to diminish when the dose of vaccine was lowered to 0.0002 cc. The degree of protection on the 3rd day was sufficient to protect against 100,000 minimal lethal doses. (When the maximal virulence of the organism 10^{-9} is used in the calculations, the protection is equivalent to 1 million minimal lethal doses.) The Type I organism produces a more marked early immunity than the Type II.

Experiment 11.—(Table XI.) The antigen used in this experiment was a virulent Type I culture exposed to a final concentration of 0.1 per cent sodium ricinoleate. Mice were inoculated with this culture intraperitoneally daily for 5 days, dose 0.2 cc. On the 5th day a virulent test culture was injected intraperitoneally.

Survival began on the 4th day, and was complete on the 6th. The use of sodium ricinoleate did not hasten the immunologic response. The total results were not as good as those which were produced by the use of the serum vaccine.

DISCUSSION.

The experiments reported in the present paper are concerned mainly with (1) the character of the pneumococcus antigen as a factor in the antibody response, (2) the onset and rate of development of immunity to the pneumococcus.

As reviewed in the early part of this paper, some observers have found the intact bacterial cell necessary for the development of type-specific immunity, whereas others have reported that solutions or extracts of the pneumococcus are adequate for this purpose. We have employed four antigens, two of them consisting of the intact cell, namely, the serum vaccine and the washed bacteria vaccine; and two of them containing water-soluble substances derived from the cell, namely, a Berkefeld filtrate of the shaken bacteria and a filtrate of the broth culture. The latter antigens are contained in a clear water solution free from formed elements.

Mice were actively immunized by a single intraperitoneal injection of each antigen. On the 5th day after the injection a high grade type-specific immunity was demonstrated in all. Survival of the mice to a virulent culture of the homologous organism was not deemed sufficient

to prove type-specific immunity, for it appeared possible that the increased resistance might be due to augmenting the natural defensive mechanisms or to the stimulation of the common protein antibody. Tillett (26) in this connection found that immunization of rabbits with Type I or Type II pneumococci, and with R forms derived from any of the fixed types, was equally effective in producing active immunity against Type III infection. All the mice, therefore, that survived injection of Type II culture after previous vaccination with Type II antigen were injected with virulent Type I culture. None survived. Wright (27) was also unable to demonstrate increased resistance to Type I pneumococci by previous injection with heterologous organisms.

After a single intravenous injection to rabbits of each of the four antigens described above, all sera on the 5th day showed type-specific protective substance. The sera which protected mice against Type II culture gave no protection against Type I culture. Thus, in both active and passive immunity experiments a Berkfeld filtrate containing a watery extract of the pneumococcus cell resulted in a type-specific antibody response.

The onset of definite type-specific immunity, both active and passive, appeared on the 3rd day after injection of the serum vaccine. It increased progressively to the 5th day and remained approximately unchanged to the 7th day or longer. The washed bacteria vaccine resulted in a similar slightly less marked response. The immunity resulting from the filtrate of the shaken bacteria and the broth filtrate began on the 4th day after injection, increased on the 5th, and remained stationery from the 5th to the 7th day. The degree of active immunity secured on the 5th day from Pneumococcus Type II vaccine was approximately that which protected a mouse from an intraperitoneal injection of 0.001 cc. of a culture with a virulence of 10–6 cc. In the case of Type I vaccine protection was obtained against 0.001 cc. culture with a virulence of 10–7 cc. The degree of antibody response cannot be measured accurately solely by recording survival after injection of varying doses of test culture, for as pointed out earlier in the paper and as observed by Neill and Gaspari, the incidence of infection between the range 10–3 and 10–6 is not wholly dependent upon dilution. We have considered as of almost equal

importance the total number of animals surviving of the entire group (10–3 to 10–6) irrespective of the dose administered. Lastly, varying the virulence of the organism was of especial help in determining the graded increase of early immunity. When active immunity was tested against a Pneumococcus Type II organism of moderately high virulence, (10–6), well marked specific immunity appeared on the 3rd day. With an organism of still higher virulence (10–7) an immune response was noted on the 4th day. In two instances, when a test culture of low virulence was employed, evidence of active immunity appeared on the 2nd day. (Test culture was fatal to a mouse, 10–5 cc. in 130 hours.) In the case of Type I pneumococcus vaccine, active immunity appears on the 3rd day even against a highly virulent 10–7 organism.

By evaluating these three criteria of resistance, we feel justified in stating that an antigen derived from the intact cell gives rise to a type-specific immunity both active and passive beginning definitely on the 3rd day, increasing to the 5th, and remaining approximately stationery to the 7th or longer. The antigen contained in the filtrate, free from formed elements, initiates a type-specific immunity on the 4th day, increases markedly to the 5th, and remains approximately stationery to the 7th day. The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II. On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses. On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

We are unable at this time to evaluate the factors which are responsible for the early induction of immunity to the pneumococcus. In the preparation of our vaccine we have employed highly virulent cultures, relatively short incubation periods, and the addition of human serum to the broth. The final vaccine was cleared of broth by carefully rinsing the centrifuge tube containing the sedimented bacteria instead of washing, since the washings contained considerable highly antigenic material. The average dose of vaccine administered to the rabbit was 2 to 5 billion organisms intravenously; to the mouse 200 to 400 million organisms intraperitoneally.

This study was undertaken with the object of determining whether

an active immunity to the pneumococcus could be established in a sufficiently short space of time as to make the injection of vaccine a therapeutic possibility in lobar pneumonia. As far as the time interval is concerned, our results support this hypothesis. Whether the patient with lobar pneumonia would react as experimental normal animals do raises a question not within the scope of this paper.

CONCLUSIONS.

1. The antigenic function of a pneumococcus vaccine made from the intact cell was compared with that derived fron a watery extract of the cell free from formed elements. In each instance, the immunity produced was dependent upon type-specific protective substance and not upon the elaboration of the common protein antibody.

2. The vaccine made from the intact cell resulted in both active and passive immunity which began on the 3rd day, increased markedly to the 5th, and remained approximately stationery to the 7th day. In the case of the Berkefeld filtrate of the shaken bacteria and the filtrate of the broth culture, the immunity began on the 4th day, increased to the 5th, and remained approximately stationery to the 7th day. The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II. On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses. On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

3. Certain factors related to the preparation and dosage of the vaccine are discussed.

4. As far as the time interval and the degree of immunity produced are concerned, these results suggest the possibility of employing pneumococcus vaccine in suitable doses in the treatment of lobar pneumonia. That an earlier activity of the immunity mechanism could actually be initiated in a patient with lobar pneumonia has still to be demonstrated.

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THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

III. The Malignant Fibroblast of Sarcoma 10 of the Crocker Foundation.

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Plate 4.

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The definition of a cell by its morphological characteristics, according to classical cytology, is of an unsatisfactory nature because the individuality of an anatomical element depends more on its physiological properties than on its appearance.[1] A description of a given structure remains almost without significance if the relations that correlate its form and function remain unknown. Such a study should always be completed by a thorough investigation of the fundamental physiological properties of the element considered. In this manner, the individuality of the fibroblast and the macrophage has become clearly defined;[2,3] but an investigation of the sort must be undertaken for every cell type that has been obtained in pure culture. If a similar study were made of the cells composing experimental tumors, the nature of malignancy might soon be discovered. A search has already been started for the essential characteristics of the specific elements of Rous sarcoma,[4] of rat sarcomas,[5] and of mouse and rat carcinomas,[6] with the object of ascertaining the factors which cause

[1] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1198. Carrel, A., in Cowdry, E. V., Special cytology, New York, 1928, 1.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 261.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 285.

[4] Carrel, A., *Compt. rend. Soc. biol.*, 1925, xcii, 584.

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1119.

[6] Fischer, A., *Z. Krebsforsch.*, 1927, xxv, 89. Laser, H., *Z. Krebsforsch.*, 1927, xxv, 298.

these cells to multiply indefinitely within the organism. The purpose of the present paper is to describe the properties that distinguish the malignant fibroblast of Sarcoma 10 of the Crocker Foundation from the normal fibroblast of the rat, when both cell types are living in pure cultures.

Isolation of a Strain of Malignant Fibroblasts.

Rat Sarcoma 10, which was obtained by us from the Crocker Foundation through the kindness of Dr. F. C. Wood, is a tumor easily transmissible by transplantation. Its growth is rapid. It spreads locally, reaches a very large size, and kills the animal by cachexia. There are no metastases. Regression of the tumor is exceptional. It is composed of large, short spindle cells with oval nuclei and one or two nucleoli, densely packed together. Between the cells are scattered many macrophages which may easily be distinguished from the fibroblasts by the appearance of their nucleus. When a pure culture of malignant fibroblasts is inoculated into a rat, the tumor which develops after less than 5 days already shows many macrophages mixed with the spindle cells. The macrophages thus appear to be a normal constituent of the tumor.

In November, 1926, a few small fragments of Sarcoma 10 were cultivated in D flasks containing a solid medium composed of chicken plasma. When chicken plasma is diluted with 3 volumes of Tyrode solution, coagulated with 1 drop of chick embryo juice, and washed once or several times in an excess of Tyrode solution, it yields a coagulum which is not toxic for foreign cells.[7] This coagulum has the advantage of remaining transparent and of not being digested by the rat tissues.

Some diluted chick embryo juice was injected at the surface of the coagulum as a nutrient medium, for it is known that rabbit, guinea pig, rat, fowl, and other animal tissues, utilize foreign embryo proteins for the building up of new protoplasm.[8] During the first 24 hours of incubation, the tissue fragments surrounded themselves with a crown of macrophages. The migration of these macrophages was more abundant in a nutrient medium composed of heparinized rat plasma or of rat serum, than when embryonic juice was used. In rat serum or heparinized plasma, the macrophages spread rapidly

[7] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 601.

[8] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 603. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 499.

throughout the medium. But in a nutrient medium composed exclusively of chick embryo juice, they grew very much less extensively, while the fibroblasts rapidly increased in number. Every 2 or 3 days, after the cultures had been washed in Tyrode solution, the nutrient medium was changed. When the tissues had grown for about 10 days, the solid medium was removed from the flask, spread on a glass plate, and the area of growth isolated by four sharp cuts of a cataract knife. It was then divided into two parts and placed in a fresh flask. After a few passages in embryo tissue juice, all the macrophages disappeared, and a pure strain of large, short fibroblasts, similar to those seen in the sections of the tumor, was obtained. The morphology of the cells varied considerably according to the medium. They often appeared as long and densely packed fibroblasts which invaded the solid medium as a thick tissue. The rate of growth was measured by the ordinary technique.[9]

From time to time, a few fragments of cultures were inserted in the subcutaneous connective tissue of rats. 4 or 5 days after inoculations made in December, 1926, when the strain of fibroblasts was already free from macrophages, a small tumor appeared at the site of injection, grew rapidly, and eventually killed the animals. The fibroblasts evidently carried the malignant characteristics. Similar experiments were done later, with identical results. In January, and February, 1928, inoculations of the cultures still produced tumors within 4 or 5 days, which grew rapidly afterwards. There is, then, no doubt that malignancy is a permanent property of the strain.

A pure strain of normal fibroblasts was obtained from the rat by the ordinary technique.[9] It was cultivated under the same conditions as the sarcomatous cells, and used as a means of comparison. The morphological and physiological properties of the normal and malignant strains were studied in the same manner as those of the normal chicken fibroblasts described in a previous article.[2]

Morphological Characteristics of the Cells.

The normal and sarcomatous fibroblasts were compared after they had been cultivated for a few days in identical media, and when their

[9] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

rates of growth had become similar. Such preliminary conditions were necessary since it has been found that the nuclear and protoplasmic structures of normal fibroblasts vary in a large measure according to their metabolic state.[2] Were the functional state of the cells not ascertained, phenomena due to nutritional or degenerative changes might be falsely attributed to pathological factors.

In the following experiments, the normal and sarcomatous cells respectively were cultivated for several days in a medium composed of embryo tissue juice. Then, a few fragments of the cultures were transferred to hanging drops of plasma and embryonic juice. From 1 to 48 hours after the cover glasses were prepared, the tissues were stained with 1/20,000 Janus green and 1/50,000 neutral red. Camera lucida drawings were made of the cells at a magnification of 1,600 to 3,200 diameters.

The normal fibroblasts of the rat closely resembled the chicken fibroblasts previously described.[2] They were elongated cells with sharp boundaries and processes open at the end. The nuclei were long and oval, with one or two nucleoli. In about 0.5 per cent of the cells, two or three nuclei were observed. The dimensions of the cells averaged approximately $30 \times 115\mu$, and those of the nuclei $8.5 \times 19\mu$. The projected areas of the cell and nucleus were, respectively, 1,960 and 189 sq. μ. The segregation apparatus was small, and localized in the forward part of the cytoplasm around the centriole. Long filamentous mitochondria were seen around the nucleus and within the processes (Fig. 1, *A*).

The malignant cells were generally larger and coarser than the normal ones, and the cytoplasm was more refringent. Their length was about 125μ, and their width 39μ. The nucleus was globular, $12 \times 18.8\mu$, and wider but a little shorter than that of the normal cells. The projected areas of the cell and nucleus were, respectively, 2,300 and 230 sq. μ. The segregation apparatus was very small. There were no degenerative vacuoles or abnormal mitoses. The mitochondria were similar to those of normal cells (Fig. 1, *B*). Multinuclear cells were present in the proportion of about 0.5 per cent. Some of these cells contained a large number of small nuclei. However, the percentage of the abnormalities did not exceed that present in normal cultures. The malignant fibroblasts apparently did not de-

generate or die when cultivated as a pure strain. They looked like healthy cells distinguished from normal fibroblasts merely by their size and the particular appearance of their cytoplasm. Sarcomatous and normal fibroblasts may indeed assume, in some cultures, an identical form. So far, no morphological characteristic has been discovered which can be considered as specific of malignancy.

The cinematographic records of normal fibroblasts of the rat showed that their mode of locomotion is identical with that of chicken fibroblasts. The cells generally move out from the center of the colony in a straight line. Their activity is polarized, the outer pole being characterized by the presence of the centriole. The protoplasm streams through the apparently rigid walls of the open end of the front process. Then the nucleus and the cytoplasmic organs glide forward, dragging the rear process. Sarcomatous fibroblasts showed no essential differences, even when both normal and malignant strains were cultivated in the same medium and cinematographed simultaneously.

Architecture of the Colonies.

Normal fibroblasts never grow as isolated units. They do not scatter through the medium as macrophages do. When they migrate into the surrounding coagulum, they remain in intimate reciprocal contact on all sides and multiply actively when packed together. A fibroblast colony always forms a dense tissue. This characteristic establishes a fundamental difference between fibroblasts and macrophages. Macrophages live as independent units and die if they congregate in masses. Normal rat fibroblasts form round or oblong colonies, which are similar in appearance to those of chicken fibroblasts. They never invade the entire medium. When a small colony of rat fibroblasts is cultivated in a washed coagulum of chicken plasma in a D flask, and fed upon diluted chick embryo juice at a pH of about 7.4, it reaches a diameter of about 8 or 10 mm. in 5 or 6 days. Later, the rate of growth decreases and the cells have a tendency to degenerate. Under the present conditions, in order to keep its activity, the strain must be transferred to a fresh coagulum at the end of 6 days. The limitation in the size of the colonies is probably due to the same cause which also prevents chicken fibroblasts from invading the entire medium. In the colonies of rat fibroblasts, a thick center forms more often than is the case with

chicken fibroblasts, and degeneration begins unless the necrotic tissue is removed with a cataract knife. There is no real difference between the colonies of normal rat and chicken fibroblasts.

The colonies of sarcoma fibroblasts are similar to those of the normal type. The cells never scatter into the medium. They organize as a tissue and form round or oblong colonies. These colonies are thicker than those of normal fibroblasts and are easily recognized on account of this trait. They also reach a greater size, as they actively invade the medium for 10 or 12 days. Then, their diameter may be 15 or even 20 mm. They differ from the colonies of normal fibroblasts by their large surface and their greater opacity, and not by their architecture.

Cultures of normal and of sarcomatous fibroblasts which were placed side by side in a flask exchanged cells freely. Cinematograph records taken at the beginning of the symbiosis clearly showed that normal cells were not repelled or destroyed by the malignant ones. At this time, there was no morphological difference between the two types of cells. After a few days, composite colonies were obtained made up of normal and tumor tissues. These colonies were divided and transferred to flasks, and both types of cells, which had become easily distinguishable morphologically, were observed to migrate into the medium. But after some time, the sarcomatous tissue progressively invaded the normal tissue, which ultimately disappeared almost completely.

Residual Growth Energy.

It is well known that the inherent growth energy of a fragment of fresh tissue or of a pure culture of tissue cells can be measured by its residual energy,[10] that is, by the duration of the life of the cells and the activity they display when deprived of nitrogenous food in a medium composed of Tyrode solution. The residual energy of tissue cells probably depends on their capacity for accumulating food material while being cultivated in a nutrient medium. A comparison was made between the residual energy of colonies of normal and sarcomatous fibroblasts after they had lived for some weeks in embryonic tissue juice. Six experiments were performed, as reported in Table I.

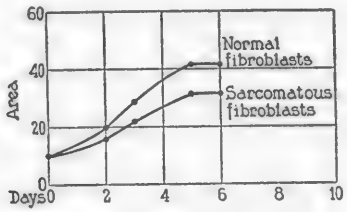
[10] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 521.

Text-fig. 1 expresses the results of an experiment which is typical. The duration of life of both normal and sarcomatous fibroblasts in Tyrode solution did not exceed 6 days. The relative increase of the normal colonies was slightly greater than that of the sarcomatous ones. The residual growth energies of both cell types were approximately equal.

TABLE I.

Effect of Tyrode Solution on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Normal fibroblasts		Sarcomatous fibroblasts	
		Duration of life	Relative increase in Tyrode solution	Duration of life	Relative increase in Tyrode solution
		days		*days*	
1	1513-H	5	0.95	5	1.40
2	1513-H	5	2.39	5	1.80
3	1517-H	9	4.30	9	1.58
4	6499-C	5	2.96	5	2.15
5	6499-C	5	3.45	5	3.07
6	6512-C	4	2.44	5	1.23
Average.....		5.5	2.75	5.67	1.87



TEXT-FIG. 1. Experiment 6499-C. Residual activity of normal and sarcomatous fibroblasts of the rat.

Duration and Rate of Growth.

When cultivated in a nutrient medium, normal rat fibroblasts multiplied in an unlimited manner, as chicken fibroblasts do. After about 16 months of life *in vitro*, sarcomatous fibroblasts proliferated as actively as at the beginning of their period of cultivation. They had also kept their malignancy. The duration of life *in vitro* of sarcomatous and normal fibroblasts appears to be unlimited.

The rate of growth of both strains has been ascertained by the measurement of the area of the colonies, and also by the volume of the new tissue which develops in a medium composed of chick embryo juice containing about 10 mg. of nitrogen per 100 cc. In Table II, the results of nine experiments are summarized, in which the rates of growth of normal and sarcomatous fibroblasts were compared for periods of 5 or 6 days. The ratio of the relative increases of the colonies in nutrient and non-nutrient media was slightly larger in the case of the normal fibroblasts. There was no fundamental difference in the rate of growth of both types of cells, as long as they were cultivated in a solution containing embryo proteins.

Effects of Normal and Sarcomatous Fibroblasts on Their Medium.

1. Liquefaction of Fibrin.—When normal and sarcomatous cells were cultivated in washed chicken plasma, no digestion of the coagulum ever occurred. The medium always remained homogeneous. When cultivated in a coagulum of rat plasma, normal rat fibroblasts did not liquefy the fibrin (Fig. 2). But if sarcomatous fibroblasts were cultivated in rat plasma, they always destroyed the coagulum down to the glass after 4 or 5 days (Fig. 3). This phenomenon gave the medium an appearance similar to that of the cultures of Rous sarcoma.[11]

2. Acid Production.—It has been shown by Rous[12] that a fragment of tissue embedded in plasma rapidly modifies the adjacent medium which becomes acid to litmus. In order to ascertain whether sarcomatous fibroblasts produce more acid than normal fibroblasts, both cell types were cultivated in a flask containing a plasma coagulum and phenol red. This dye is less toxic than some other indicators, according to the findings of Rous,[13] and is well adapted to show the changes in the pH which might be expected to occur in such experiments. After the colonies had been embedded in 1 cc. of diluted chicken plasma, coagulated by 1 drop of embryo chick juice, and washed as usual, 1 cc. of Tyrode solution containing 0.04 per cent phenol red was injected into the flask. After half an hour, it was removed and replaced

[11] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.

[12] Rous, P., *J. Exp. Med.*, 1913, xviii, 183; *Proc. Soc. Exp. Biol. and Med.*, 1911–13, ix–x, 161.

[13] Rous, P., *J. Exp. Med.*, 1925, xli, 451.

with embryo tissue juice containing 0.02 per cent phenol red. After a few hours, the sarcomatous colonies appeared golden yellow, while the normal tissues were pinkish orange. The cultures were allowed to grow for a few days. Normal and sarcomatous fibroblasts multiplied at about the same rate. Although they were in practically identical metabolic conditions, the sarcomatous tissues became bright yellow, while the normal colonies remained pinkish orange. When normal and sarcomatous tissues, instead of being cultivated as separate units, were caused to live in symbiosis, a heterologous tissue made up of patches of both cells was obtained. After the colony had been stained with phenol red, golden yellow spots appeared on a pink background. The examination of the flasks under low power showed that the yellow islands were composed of sarcomatous fibroblasts which could easily be distinguished from the normal cells by their coarser appearance. As might be expected in the light of Warburg's experiments,[14] sarcomatous fibroblasts produced much more acid than normal fibroblasts, when both cell types were placed in identical metabolic conditions. They surrounded themselves with a yellow area which extended more or less into the pink coagulum. They appeared to thrive in a pericellular fluid more acid than that about normal fibroblasts.

Optimum H Ion Concentration of the Medium.

The optimum H ion concentration of the medium was found to differ slightly for the two cell types. The sarcomatous fibroblasts must be cultivated in a well buffered medium at a pH of 7.5. The normal rat fibroblasts grow better at a pH of about 7.3 to 7.4. This slight difference is probably connected with the large quantity of acid produced by the sarcomatous fibroblasts.

Food Requirements.

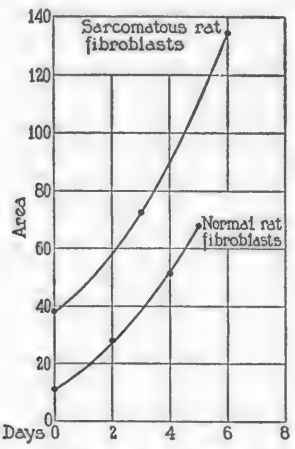
When both cell types were cultivated in chick embryo juice, they multiplied indefinitely and their rate of growth was about equal, as shown in the nine experiments summarized in Table II. The growth of the normal and sarcomatous fibroblasts of a typical experiment is expressed in Text-fig. 2. In calf liver digest, they behaved in a differ-

[14] Warburg, O., *Naturwissenschaften*, 1927, xv, 1.

TABLE II.

Effect of Tyrode Solution and Chick Embryo Juice on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$	Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$
		days				*days*			
1	6363-C					4	1.72	3.84	2.24
2	6363-C					4	1.50	2.50	1.66
3	6363-C					4	1.02	2.25	2.25
4	6425-C					5	1.25	4.03	4.03
5	6414-C	6	1.51	5.88	3.89				
6	6414-C	6	2.11	6.26	2.97				
7	6438-C	5	2.10	5.01	2.38				
8	6463-C					5	0.93	2.62	2.81
9	6463-C					5	0.90	3.42	3.80
Average.....		5.67	1.91	5.72	3.08	4.5	1.22	3.11	2.80



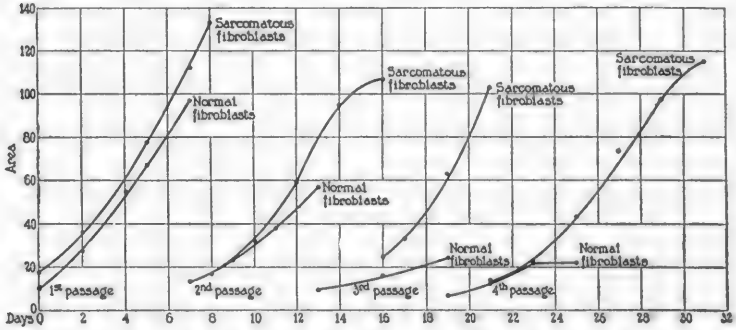
TEXT-FIG. 2. Experiments 6487-C and 9907-D. Growth of normal and sarcomatous fibroblasts of the rat in chick embryo juice.

TABLE III.

Effect of Chick Embryo Juice and Calf Liver Digest on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts [15]				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$	Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$
		days				*days*			
1	6418-C	5	3.22	3.66	1.13				
2	6418-C	5	3.94	3.69	0.93				
3	6418-C	5	3.14	3.97	1.26				
4	6445-C	12	3.99	3.04	0.69				
5	6445-C	13	4.85	3.65	0.75				
6	6445-C	13	3.67	3.26	0.89				
7	6420-C					8	5.00	6.50	1.30
8	6420-C					8	4.40	6.63	1.50
9	6451-C					16	4.90	5.20	1.06
10	6451-C					16	5.35	5.14	0.97
11	6488-C	7	9.20	11.02	1.20				
12	6488-C	7	8.72	8.30	1.05				
13	6479-C					21	2.04	2.22	1.09
14	6479-C					21	1.38	3.07	1.38
15	6507-C					28	5.00	6.00	1.20
16	6505-C					31	5.64	7.00	1.24
17	6505-C					31	6.17	8.31	1.34
18	6517-C	13	3.74	3.04	0.81				
19	6538-C	19	2.68	1.10	0.45				
20	6538-C	19	4.27	1.44	0.34				
21	6553-C	23	4.38	1.92	0.44				
22	6553-C	23	5.97	1.20	0.20				

[15] The figures express the relative increase of the tissue during the last passage, and not during the entire period of growth.



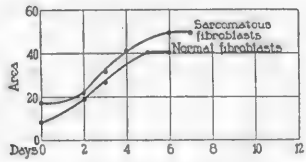
TEXT-FIG. 3. Experiments 6420-C and 6488-C. Effect of calf liver digest on normal and sarcomatous fibroblasts of the rat.

ent way. Sarcomatous fibroblasts, studied in the twenty-two experiments summarized in Table III and in Text-fig. 3, multiplied more actively in calf liver digest than normal fibroblasts did. The duration of their activity in calf liver digest was unlimited, while the normal cells did not multiply in the same medium for more than 3 or 4 weeks

TABLE IV.

Effect of Tyrode Solution and Chicken Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$	Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$
		days				*days*			
1	6477-C					5	1.32	2.00	1.51
2	6478-C					7	1.00	1.52	1.52
3	6493-C					14	1.59	1.44	0.90
4	6486-C	4	2.70	3.50	1.30				
5	6486-C					4	0.80	1.24	1.55
6	6499-C	5	3.45	3.60	1.04				
7	6499-C					5	3.07	3.04	1.10
8	6512-C	10	2.44	3.22	1.32				
9	6512-C					10	1.23	1.10	0.89



TEXT-FIG. 4. Experiments 6486-C and 6499-C. Effect of chicken serum on normal and sarcomatous fibroblasts of the rat.

despite a few transfers. Calf liver digest fulfilled all the food requirements of sarcoma cells, but it failed to support the indefinite proliferation of normal rat fibroblasts.

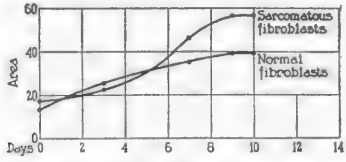
The effect of chicken serum on both cell types was ascertained in nine experiments (Table IV). In it the duration of life of both normal and sarcomatous fibroblasts did not exceed 8 days (Text-fig. 4). It is

evident that the cells do not utilize chicken serum. A similar investigation was carried out in seven experiments with rat serum (Table V). Normal and sarcomatous fibroblasts did not feed on rat serum proteins. They died after less than 7 days (Text-fig. 5.) Were it not for the observations with calf liver digest, one might suppose sarcom-

TABLE V.

Effect of Tyrode Solution and Rat Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$	Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$
		days				*days*			
1	1513-H	5	0.95	1.03	1.08				
2	1513-H	5	2.39	1.90	0.80				
3	1513-H					5	1.40	2.00	1.42
4	1513-H					5	1.80	1.82	1.00
5	6506-C					5	1.60	2.10	1.31
6	1517-H	9	4.30	1.90	0.44				
7	1517-H					9	1.58	2.18	1.40
Average.....		6.33	2.55	1.61	0.77	6	1.60	2.03	1.28



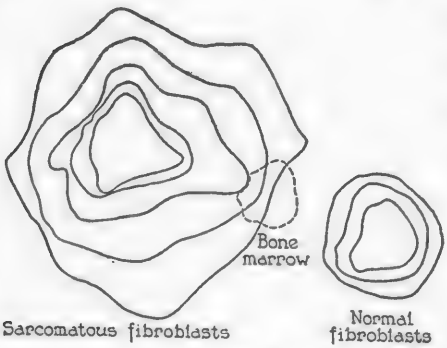
TEXT-FIG. 5. Experiment 1517-H. Effect of rat serum on normal and sarcomatous fibroblasts of the rat.

atous fibroblasts to have the same food requirements as normal fibroblasts.

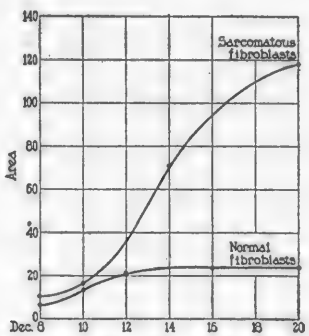
Effect of Bone Marrow on Normal and Sarcomatous Fibroblasts.

In a first series of experiments, cultures of sarcomatous and normal fibroblasts were divided into two equal parts. At an equal distance from two half cultures of malignant and normal fibroblasts, a little

fragment of rat bone marrow was placed. The medium contained diluted rat serum, and no embryonic juice. The other halves of the sarcomatous and normal colonies were cultivated as controls in a flask which contained rat serum and no bone marrow. The tracings of the tissues were made under the projectoscope and the growth of each fragment was ascertained during successive days (Text-fig. 6). The results of the four experiments of this series were constant. The ameboid cells which spread from the bone marrow multiplied more abundantly around the sarcomatous than the normal colony. The growth of the sarcomatous fibroblasts became far greater than that of



TEXT-FIG. 6.



TEXT-FIG. 7.

TEXT-FIG. 6. Experiment 1492-H. Effect of rat bone marrow on sarcomatous and normal fibroblasts of the rat in rat serum.

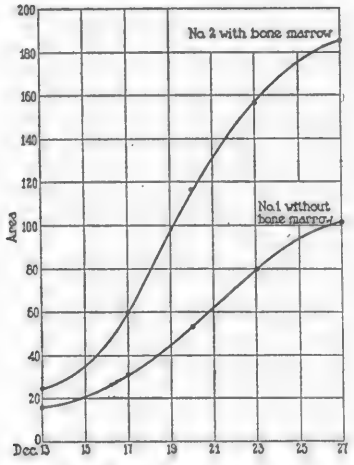
TEXT-FIG. 7. Experiment 1492-H. Effect of rat bone marrow on normal and sarcomatous fibroblasts of the rat in rat serum.

the normal (Text-figs. 6, 7). Both the controls in rat serum without bone marrow grew at equally slow rates and for only a short time. It was obvious that the multiplication of the sarcomatous fibroblasts was markedly favored by the presence of the bone marrow, while the normal fibroblasts responded but slightly. In a second series of experiments, a culture of sarcomatous fibroblasts was divided into two parts. One fragment was cultivated in rat serum without bone marrow, and the other with bone marrow. The fragment located close to the bone marrow grew faster than the isolated one (Text-fig. 8). The

sarcomatous fibroblasts apparently received from the ameboid cells of the bone marrow, directly or indirectly, certain substances which promoted their multiplication.

SUMMARY AND DISCUSSION.

It is an important fact that, after many months of life *in vitro*, in a medium composed exclusively of chicken plasma and embryonic juice or calf liver digest, the strain of fibroblasts isolated from Sarcoma 10 of the Crocker Foundation had kept its malignancy unimpaired. The indefinite persistence of this characteristic in a pure strain of



TEXT-FIG. 8. Experiment 1518-H. Effect of rat bone marrow on sarcomatous fibroblasts of the rat.

mammalian tissue renders possible, under ideally precise conditions, a study of the properties which are specific to malignancy. It opens a new era in the investigation of experimental cancer of mammals. By a method similar to that here described, Fischer and Laser have isolated the active elements of Ehrlich and Flexner-Jobling carcinomas,[6] and we have recently obtained pure cultures of the Jensen sarcomatous fibroblasts. There is little doubt that the malignant constituents of other experimental tumors can be isolated in the same

manner, and the properties associated with their unlimited growth within the body discovered.

The pure strain isolated from Sarcoma 10 is composed of fibroblasts. The cells possess all the morphological characteristics of their type. Their mode of locomotion does not differ from that of the normal fibroblasts. Their colonies are built in the same manner. However, they are larger and coarser than the normal fibroblasts of the rat when observed in pure culture (Fig. 1, *A*). Possibly, there is some other morphological difference that has not been detected by our methods of examination, such as an increase in the number of chromosomes or a change in their shape or volume. No abnormal mitoses or inclusions in the nucleus or the cytoplasm have been observed. When cultivated in a pure state, the sarcomatous fibroblasts are free from the secondary factors which *in vivo*, as well as in the mixed cultures of fresh tumor, may alter their appearance. They never degenerate and die, but multiply indefinitely. To all appearances, they are healthy cells. The malignancy that they display within the body must not be attributed to a diseased condition, but to the presence of some new physiological property.

The sarcomatous fibroblasts possess no more inherent growth energy than the normal ones. Both cell types show approximately the same residual energy in Tyrode solution and the same duration and rate of growth when placed in a nutrient medium. Like normal fibroblasts, the malignant cells multiply indefinitely in chick embryo juice, and die after a few days if cultivated in chicken or rat serum. However, they differ from the normal cells in one of their food requirements. If cultivated in a medium composed exclusively of calf liver digest, they grow in an unlimited manner, while normal fibroblasts in such a medium die within a few weeks. Although anarchical in behavior within the body, sarcomatous fibroblasts living *in vitro* require for their proliferation conditions which are almost identical with those demanded by the normal type.

However, they differ sharply from the normal fibroblasts in two aspects: they liquefy coagulated rat plasma, while normal fibroblasts do not, and they constantly produce more acid than normal fibroblasts placed under similar metabolic condition. The dissolution of the fibrin of the solid medium shows the cultures to possess the same characteris-

tics as cultures of Rous sarcoma macrophages.[11] The coagulum assumes a striking, moth eaten appearance. The increased acid production is an expression of the phenomenon studied in the well known experiments of Warburg.[14] If the liquefaction of the fibrin is due to its digestion, the malignant cells should be considered as setting free more active proteolytic enzymes than do normal ones. As proteoses and peptones are known to cause cell proliferation,[16] an increased peptic secretion, or an enhancement of the normal peptic activity by acid production, would explain the mechanism of unlimited growth. But the cells of Sarcoma 10 do not appear to hydrolyze serum proteins, or to obtain from them the required proteoses and peptones, since they do not proliferate in a serum medium. Therefore, it is difficult to understand how they multiply within the body more actively than normal fibroblasts.

Although interstitial lymph may be assumed to possess no more nutrient properties than diluted plasma, an attempt was made to ascertain whether it contains any growth-promoting substance for Sarcoma 10. A pure culture of sarcoma cells was grafted in the subcutaneous connective tissue of a rat for 24 hours, then removed, and cultivated in a flask. Its growth energy was found to have decreased considerably, and was recovered only after several days of life in chick embryo juice. But, when the culture was allowed to stay within the rat for 4 or 5 days, a small tumor appeared. Fragments of such a tumor cultivated in flasks immediately became surrounded with a crown of emigrated macrophages. It was obvious that the pure culture of sarcomatous fibroblasts, during its short stay in the rat, had attracted macrophages from the subcutaneous connective tissue. These macrophages are a normal constituent of the tumor *in vivo*. It may be supposed that the sarcomatous fibroblasts are supplied by the macrophages, directly or indirectly, with certain substances which are growth-promoting if acted upon by ferments. It is known that in normal tissues, macrophages may have such a nutritive function. Renaut thought that the lymph cells bring to fixed cells some food that they need.[17] Later, it was found that macrophages effectively

[16] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.
[17] Renaut, J., *Arch. anat. micr.*, 1906–07, ix, 495.

promote the proliferation of normal fibroblasts.[18] It is possible that the growth within the body of Sarcoma 10 is due to a similar phenomenon.

This hypothesis has been submitted to experimental test, and the effect of living cells on normal and sarcomatous fibroblasts cultivated in rat serum has been investigated. When a fragment of rat bone marrow was placed between two colonies of normal and malignant fibroblasts, equidistant from both, the malignant fibroblasts proliferated far more actively than the normal ones. At the same time, the wandering cells migrating from the bone marrow became more numerous in the acid area surrounding the sarcoma than in the normal tissue. It was obvious that, directly or indirectly, the wandering cells brought to the sarcomatous fibroblasts the substances required for multiplication. Instead of being the expression of a defensive reaction, the macrophages of Sarcoma 10 may supply the fibroblasts with proteins such as exist in embryo juice or with protein split products which determine their unlimited proliferation within the organism. One may suppose that the growth of Sarcoma 10 depends on the simultaneous presence of two elements: the specific malignant cells, and the nursing macrophages.

CONCLUSIONS.

1. A pure strain of fibroblasts has been isolated from Sarcoma 10 of the Crocker Foundation. After about 16 months of life *in vitro*, the malignancy of the strain is as great as that of the original tumor.

2. The strain has been compared with a strain of normal rat fibroblasts. The malignant cells are generally larger, coarser, and more refringent than normal cells. They possess all the morphological characteristics of fibroblasts. They do not show any abnormalities and never degenerate and die. They are to all appearances healthy cells. Their mode of locomotion is identical with that of normal fibroblasts. Their colonies are larger, but the architecture is similar.

3. The residual activity of both cell types, the duration of their life, and their rate of growth in a nutrient medium are almost identical.

4. The sarcomatous fibroblasts liquefy a rat plasma coagulum while

[18] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645. Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.

normal fibroblasts do not. They turn phenol red golden yellow whereas, under the same conditions, normal cells turn it pinkish orange.

5. Sarcomatous and normal fibroblasts of the rat multiply to an unlimited degree in chick embryo juice. They live for only a short time in rat serum and chick serum. Calf liver digest will suffice for an unlimited proliferation of sarcoma fibroblasts, but fails to support the life of normal fibroblasts for very long.

6. The presence of bone marrow greatly increases the rate of growth of sarcomatous fibroblasts cultivated in rat serum, while it only slightly affects that of the normal cells. The unlimited growth of the sarcomatous tissue in animals to which it is transplanted may be attributed to the presence of macrophages, which are a normal constituent of the tumor, and possibly are a necessary factor of its growth *in vivo*.

EXPLANATION OF PLATE 4.

FIGS. 1, *A* and *B*. *A*, Culture 6827-C. Camera lucida drawing of a cell from a pure culture of fibroblasts from a normal rat. *B*, Culture 6795-C. Camera lucida drawing of a fibroblast from Sarcoma 10. Stained with 1/20,000 Janus green and 1/20,000 neutral red. The neutral red vesicles and granules are represented in gray, the fat globules by circles, and the mitochondria by lines.

FIG. 2. Culture 1482-H-1. Colonies of normal fibroblasts after 4 days in rat plasma coagulum.

FIG. 3. Culture 1482-H-2. Colony of sarcomatous fibroblasts after 4 days in rat plasma coagulum.

FIG. 1.

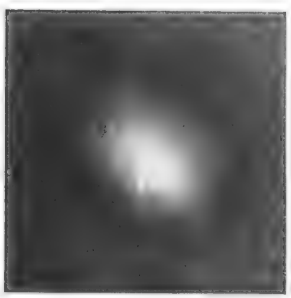


FIG. 2.

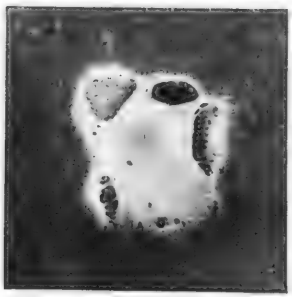


FIG. 3.

(Carrel and Ebeling: Properties of fibroblast and macrophage. III.)

RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

II. The Influence of Vaccinal Immunity on the Reaction to Experimental Syphilis.

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(Received for publication, April 20, 1928.)

In the preceding paper of this series, the effects of a concomitant vaccinal infection upon the reaction to experimental syphilis in the rabbit were reported (1). It was shown that under the conditions of the experiments which involved the intracutaneous inoculation of vaccine virus at the time of an intratesticular inoculation of *Treponema pallidum*, the syphilitic reaction was profoundly disturbed in the direction of lowered efficiency, the disease which developed being much more severe than in control animals.

The syphilitic reaction has also been studied, first, in rabbits in which both vaccine virus and *T. pallidum* were inoculated simultaneously in the same site (testicle) and, second, in rabbits which had been previously immunized to vaccine virus. The study of the sort first mentioned had its origin in the unexpected results obtained with the intratesticular inoculation of syphilitic material derived from a rabbit inoculated with a strain of *T. pallidum* reported to be capable of inducing a severe infection (1). The syphilis in our inoculated animals proved unusually mild, and it was found that the original rabbit from which the strain was derived was infected as well with vaccine virus which was being transferred in the syphilitic inocula. The purpose of the second series of experiments dealing with the syphilitic reaction in vaccine-immune rabbits was to determine whether the character of the syphilis in the animals inoculated with the material containing both *T. pallidum* and vaccine virus as mentioned above, was influenced by the vaccinal immunity, an immunity well established by the time the first clinical signs of the syphilitic infection are appearing.

A preliminary report of the findings has already appeared (2). In the present paper they are described in detail.

EXPERIMENTAL.

Material and Methods.

The experiments were carried out at the same time as others previously reported (1), the same materials being used for inoculation and in like amounts. The rabbits were young adult male animals approximately 8 months of age. They were separately caged throughout the period of observation and were fed a diet of hay, oats and cabbage.

The dates of inoculation were: Experiment I, November 10, 1926; Experiment II, January 13, 1927; Experiment III, February 14, 1927. The Nichols strain of *T. pallidum* and the Noguchi strain of vaccine virus were used, the material for inoculation in each case being obtained from actively developing testicular lesions. The emulsions prepared with the syphilitic material contained from 1 to 3 actively motile spirochetes to the microscopic field.

Control Series.—The syphilitic controls comprised 3 groups of 10 rabbits each; the same groups also served as the controls of the experiments previously reported (1). Each animal was inoculated in 1 testicle with 0.2 cc. of the *pallidum* emulsion.

The virulence of the vaccine virus was tested in each experiment by the inoculation of 2 to 5 normal rabbits. The inoculations (in each instance 0.2 cc. of a saline emulsion) were made intracutaneously on a shaved area of skin on the side of the body, as well as by application to a scarified skin area; an intratesticular injection was also made in certain rabbits.

Vaccine-Immune Series.—There were 3 groups of rabbits immunized to vaccine virus by the intracutaneous injection of 0.2 cc. of a virus emulsion. Each animal developed a typical reaction, and a second injection 10 days after the first in the case of the first and second groups and 15 days later in the case of the third group, was followed by no visible reaction. These rabbits were subsequently inoculated in 1 testicle with *T. pallidum* (0.2 cc.). The length of time between the first injection of vaccine virus and the syphilitic inoculation was 20 days in the first, 84 days in the second and 30 days in the third experiment. There were 5 rabbits each in the first and second groups and 10 in the third.

T. pallidum Plus Vaccine Virus Series.—There were 5 rabbits in the first and 5 in the second experiment in which a mixture of syphilitic and vaccinal emulsions was injected in 1 testicle. In the first experiment the mixture was prepared with twice as much vaccine as syphilitic emulsion and the inoculating dose of 0.6 cc., therefore, contained 0.4 cc. of vaccinal and 0.2 cc. of syphilitic material. In the second experiment, the mixture contained equal amounts of the 2 emulsions and each animal received 0.4 cc.

Conduct of Experiments and Analysis of Results.—The period of observation in each experiment was 3 months from the date of syphilitic inoculation. Special attention was given to the time and frequency and to the duration of successive phases of the reaction to syphilitic infection with a view to reducing the comparison of results to as quantitative a basis as possible. The particular conditions chosen

for comparison were: the incubation time of primary lesions, the time and frequency of the occurrence of a critical edema in the inoculated testicle, the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones or eyes, the number of foci affected by such lesions and finally, the proportion of animals that showed complete healing of all lesions during the 3 months observation period.

It should be noted, in discussing time relations of the various reactive phenomena, that there is a basic tendency toward the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits. With the Nichols strain of *T. pallidum* as carried in our laboratory, the reaction interval is approximately 2 weeks. In these experiments, as stated above, the Nichols strain was used, but the particular line from which the present substrain, as it may be termed, was derived had been transferred under somewhat different conditions from those employed with the parent strain. It was not known at the time this work was started, however, that the infection induced by this substrain would differ in any essential respect from that of the parent strain, but the results of these and other experiments have shown certain peculiar features of the syphilitic reaction, notably a delay in the development of a metastatic orchitis and an unusually early appearance of generalized lesions. These peculiarities in the behavior of the strain must be taken into account in analyzing the results obtained.

In recording the results, the term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions, while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparison of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

Results.

The results of the experiments are presented in Tables I, II and III and in Text-figs. 1 and 2.

The values in Tables I and II which represent group means, refer to the incidence and time of occurrence of various phenomena of the syphilitic reaction; Table III contains the total number of generalized lesions in the order of their appearance. The successive phases of the infection which to some extent overlap each other, are shown in Text-fig. 1 by a series of curves plotted upon a time basis. To facilitate the reading of these graphs, an arbitrary division of the curves has been made at the 35th day and those representing the incidence and the focal distribution rate of generalized lesions have been separated from the

TABLE I.

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions.

Experiment	No. of rabbits	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					Incidence	Focal distribution	Focal distribution
		per cent	*per cent*	*per cent*	*per cent*	*actual*	*relative*
I C	10	100.0	60.0	100.0	100.0	9.8	9.8
V.V. Im.	5*	100.0	100.0	100.0	75.0	8.7	6.5
V.V. (I.T.)	5	100.0		100.0	40.0	10.5	4.2
II C	10	100.0	50.0	90.0	70.0	7.0	4.9
V.V. Im.	5	100.0	40.0	80.0	80.0	4.0	3.2
V.V. (I.T.)	5	100.0		100.0	20.0	2.0	0.4
III C	10	100.0	70.0	90.0	90.0	8.4	7.6
V.V. Im.	10	100.0	40.0	90.0	90.0	7.1	6.4
Mean values							
C	30	100.0	60.0	93.3	86.7	8.6	7.4
V.V. Im.	20	100.0	55.5	90.0	89.5	6.3	5.6
V.V. (I.T.)	10	100.0		100.0	30.0	7.7	2.3

* There was 1 accidental death in this group shortly after the development of the metastatic orchitis.

In this and all other tables:

C = controls; V.V. Im. = animals immune to vaccine virus; V.V. (I.T.) = animals inoculated with *Treponema pallidum* and vaccine virus in the same testicle.

TABLE II.

Mean Time of Occurrence of Various Phenomena of Infection Estimated in Days from Date of Inoculation.

Experiment	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
				First	Last	Mean of all
	days	*days*	*days*	*days*	*days*	*days*
I C	12.8	31.0	46.5	48.0	78.8	62.8
V.V. Im.	9.0	28.6	50.2	57.3	79.0	66.6
V.V. (I.T.)	(67.4)*		50.8	58.8	86.0	68.9
II C	22.9	40.4	61.8	62.0	80.4	67.4
V.V. Im.	20.8	32.5	56.8	63.3	73.0	68.8
V.V. (I.T.)	(44.5)*		58.2	89.0	100.0†	95.0
III C	12.2	33.9	45.3	47.6	67.6	55.0
V.V. Im.	11.0	29.3	54.3	55.3	70.4	65.9
Mean values						
C	15.8	34.7	51.0	51.6	75.1	61.0
V.V. Im.	13.0	29.5	53.7	57.2	72.6	66.6
V.V. (I.T.)	(55.9)*		54.5	68.7	90.7	71.4

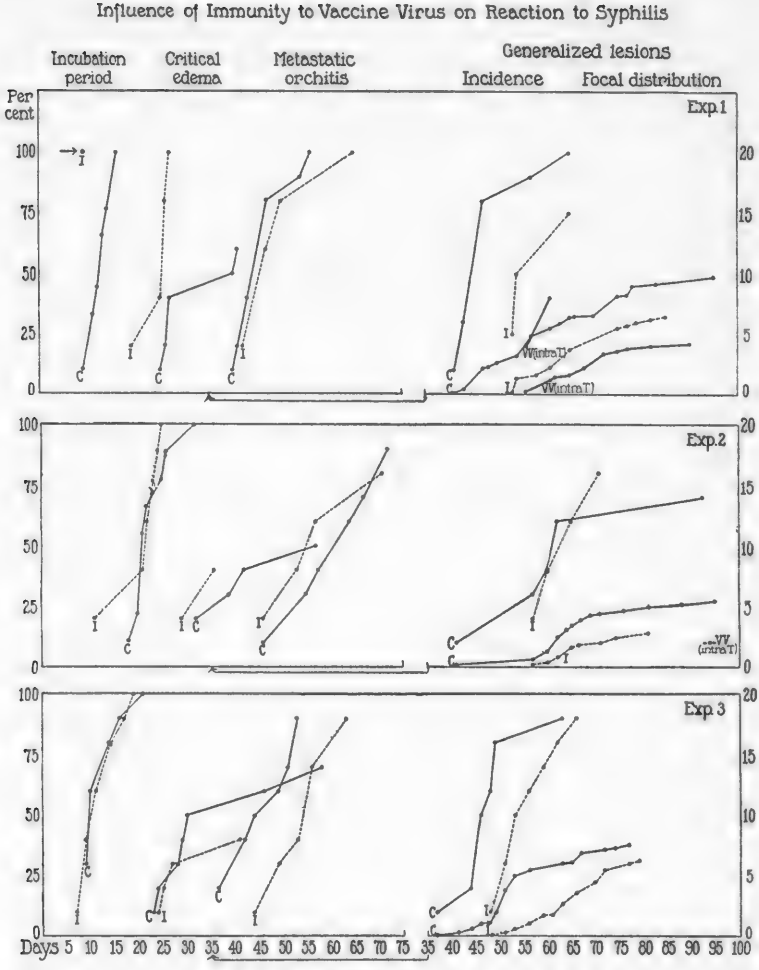
* These incubation periods are enclosed in parenthesis to indicate that they are not of the same order as the others as explained in the text.

† Since only 2 generalized lesions were detected in this group, both are included in the results, although one of them developed later than the selected period of observation.

TABLE III.

Time of Appearance of Generalized Lesions Estimated from Date of Inoculation.

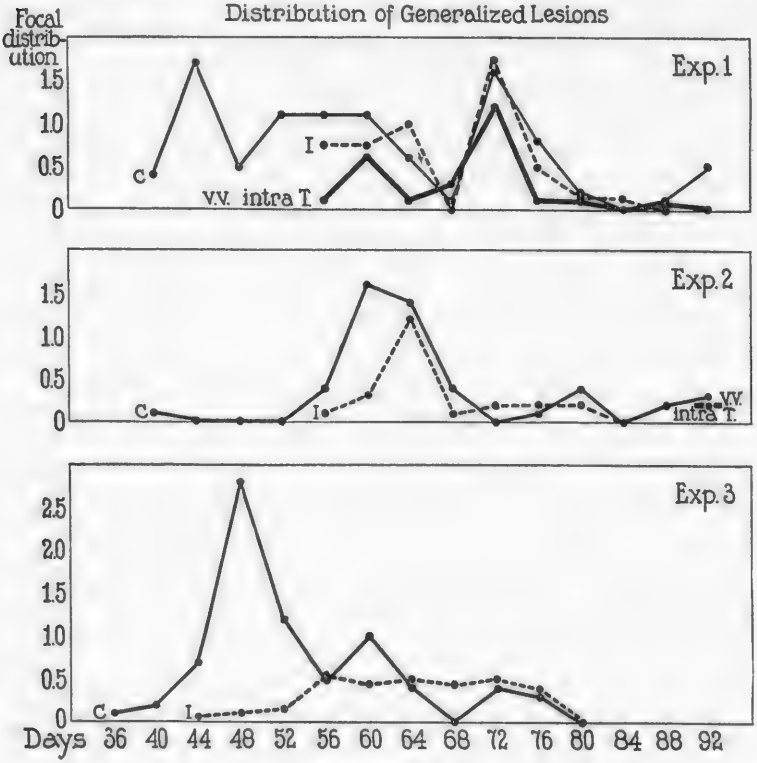
Days	Experiment I			Experiment II			Experiment III	
	Controls 10 rabbits	V.V. Im. 4 rabbits	V.V. (I.T.) 5 rabbits	Controls 10 rabbits	V.V. Im. 5 rabbits	V.V. (I.T.) 5 rabbits	Controls 10 rabbits	V.V. Im. 10 rabbits
35	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	1	0
39	0	0	0	0	0	0	0	0
41	1	0	0	1	0	0	2	0
43	3	0	0	0	0	0	3	0
45	0	0	0	0	0	0	4	0
47	19	0	0	0	0	0	1	1
49	3	0	0	0	0	0	9	0
51	0	0	0	0	0	0	18	2
53	6	5	0	0	0	0	12	3
55	5	1	1	0	0	0	5	5
57	11	2	0	4	1	0	0	0
59	0	0	0	7	1	0	0	6
61	7	3	6	9	2	0	0	1
63	4	0	0	7	3	0	10	9
65	6	4	1	3	1	0	1	9
67	0	0	3	4	3	0	3	0
69	1	0	0	3	0	0	0	9
71	0	0	6	1	1	0	3	10
73	0	0	0	0	2	0	1	0
75	16	7	1	1	0	0	0	0
77	8	1	1	0	0	0	3	5
79	0	1	0	0	0	0	0	3
81	0	1	1	4	2	0	0	0
83	2	0	0	0	0	0	0	0
85	0	1	0	0	0	0	0	0
87	0	0	0	2	0	0	0	0
89	1	0	1	0	0	1	0	0
91+	5	0	0	3	0	1	0	0
Total	98	26	21	49	16	2	76	63


Influence of Immunity to Vaccine Virus on Reaction to Syphilis
Incubation period
Critical edema
Metastatic orchitis
Generalized lesions
Incidence
Focal distribution
Per cent
Exp.1
Exp.2
Exp.3
VV (intra T)
Days

TEXT-FIG. 1.

others. The curves in Text-fig. 2 which illustrate the distribution or time of appearance of generalized lesions are compiled from the values listed in Table III.

It will be noted that whereas there were 3 groups each of vaccine-immune and control animals, there were but 2 groups of rabbits inoculated with a mixture of syphilitic and vaccinal emulsions. Strictly speaking, therefore, the results of



TEXT-FIG. 2.

the doubly inoculated animals should be compared with the mean values of the corresponding 2 groups of controls and vaccine-immunes, rather than with the values of the 3 groups. It has been found, however, that there is little difference between these 2 sets of mean values, so that in order to simplify the analyses of results, only the mean value of the 3 groups has been used.

DISCUSSION.

It will aid to a clearer understanding of the results obtained in these experiments if the various phenomena of the syphilitic reaction chosen for discussion are taken up in the order of their development. In this way, one obtains a picture of the infection in its successive phases and is the better able to appreciate the significance of variations in the experimental series.

Primary Orchitis.—All rabbits in each group of the 3 experiments developed a primary lesion of the inoculated testicle, that is, the controls, the vaccine-immunes and the animals inoculated intratesticularly with a mixture of vaccinal and syphilitic viruses (Table I). The time at which the lesion was detected, however, differed in the 3 groups (Table II). The mean value of the incubation period was 15.8 days for the controls but it was slightly shorter, 13.0 days, for the vaccine-immunes. The results of individual experiments, moreover, were consistent in this respect, the difference in time being 3.8, 2.1 and 1.2 days respectively. This finding which will be discussed later in connection with other results, suggests that the character of the initial reaction of rabbits immune to vaccine virus was modified in the direction of increased susceptibility or heightened sensitivity.

In the case of the 2 groups of rabbits which were doubly inoculated, the development of a primary lesion was markedly delayed, the mean value of the incubation period being 55.9 days, a prolongation of 40.1 days beyond the mean incubation of the controls (Table II). At least 2 reasons suggest themselves as the cause of this result. In the first place, it is probable that the intense testicular reaction to vaccine virus seriously interfered with the development of spirochetes if it did not actually destroy a certain number and second, that the condition of the testicle after the subsidence of the vaccinal reaction was not such as to favor the usual growth of organisms and the production of a local lesion. In this connection, it is of interest to note that vaccine virus *per se* had no apparent immediate effect on the motility of the spirochetes as judged by successive dark-field examinations of the mixture of the 2 emulsions. Actively motile spirochetes were seen for as long as 4 hours in the mixtures kept at room temperature and in the ice box.

As will be brought out in the following discussion, the general character of the disease in any particular experiment of this nature has an important bearing on the results obtained. An idea of the variation in the disease in the 3 experiments here reported may be had by comparing the mean time at which the primary orchitis developed in the control animals. Reference to Table II shows that the incubation period in the second experiment was much longer than in the first or third, that is, 22.9 as compared with 12.8 and 12.2 days, and as will be seen, these values are indicative of the mild disease in the second and of the more severe infections in the first and third experiments. While the incubation periods of both groups of doubly inoculated animals were greatly prolonged beyond those of the controls (Table II), it was not as delayed in the second as in the first experiment, the difference in the 2 cases being 21.6 and 44.6 days respectively. This result is surprising in view of the character of the disease in the second series, for it might be thought that the effect of the vaccinal reaction upon the primary orchitis would be more marked in a mild than in a severe infection. But the dose of vaccine virus employed in the first experiment was twice as large as in the second and furthermore, the first virus was more potent than the second judging from the intracutaneous and intratesticular reactions of the control animals. It would appear, therefore, that the earlier development of the primary orchitis of the second experiment was related to a less pronounced local vaccinal reaction.

Critical Edema.—Although the phenomenon of an edema of the inoculated testicle is a variable feature of the syphilitic reaction, it is a highly significant one as it marks the end, either temporary or final, of the initial stage of the infection.

The mean incidence of edema in the control groups was 60.0 per cent as compared with 55.5 per cent for the vaccine-immune groups and with none in the doubly inoculated animals (Table I).[1] The rates for individual control groups are, on the whole, average for the Nichols strain of *T. pallidum.* The first vaccine-immune group, however, showed the high incidence of 100.0 per cent, a rate not attained by any other group in the 3 experiments. This result indicates a high

[1] The early edema of the testicle associated with the reaction to vaccine virus is, of course, not included in the present connection.

capacity for reaction on the part of these animals, and in this connection, it should be remembered that the shortest incubation period of the primary orchitis and the greatest difference in the time of this phenomenon as compared with the controls occurred with this group. The interpretation of this finding on the basis of an initial hypersensitiveness or increased reactivity is strengthened by the uniform occurrence of a critical edema.

The time at which edema occurred shows that the vaccine-immune animals reacted more promptly than the controls. That is, in the control groups, it occurred 5 weeks after inoculation and in the vaccine-immune animals approximately a week earlier, the actual mean values being 34.7 and 29.5 days (Table II). The results of individual experiments, moreover, were consistent in this respect, the differences being 2.4, 7.9 and 4.6 days respectively.

A comparison may also be made between the time of development of the critical edema and the incubation period of the primary orchitis. The mean value of this interval, as shown in the following table, was definitely shorter in the case of the vaccine-immunes as compared with the control value. As far as individual results were concerned, the reaction interval of the vaccine-immune groups in the second and third experiments was shorter than those of the controls, while in the first experiment, it was slightly longer.

From the Incubation Period to the Critical Edema.

Experiment	Controls	Vaccine-immunes
	days	*days*
I	18.2	19.6
II	17.5	11.7
III	21.7	18.3
Mean values	19.1	16.5

The observations regarding edema of the inoculated testicle show, as was the case with the incubation period of the primary lesion, that animals which had been immunized to vaccine virus reacted more promptly to an intratesticular inoculation of *T. pallidum* than did normal rabbits.

Metatastic Orchitis.—The next phenomenon of the syphilitic reaction

to be considered is the development of a lesion in the uninoculated testicle.

As shown in Table I, the incidence of a metastatic orchitis was the same in the vaccine-immune and the control groups, the mean values being 90.0 and 93.3 per cent. In the first experiment in which the severity of the disease was marked, all animals of both groups developed a metastatic orchitis, while in the second and third in which the disease was less severe, there was 1 rabbit each in both the control and the vaccine-immune groups in which a metastatic orchitis was never detected. On the other hand, the incidence of a lesion in the uninoculated testicle of the rabbits which received the double inoculation of *T. pallidum* and vaccine virus was 100.0 per cent. The fact that a metastatic orchitis developed in all doubly inoculated animals of the first experiment is not surprising in view of the severity of this particular infection. But in the second experiment, although the disease was mild, a similar result obtained, and this finding is in harmony with the comparative shortness of the primary incubation period which was discussed above. It is possible that the factor of individual animal variation may account for this result, and it is also possible that it is due to the variability of reaction phenomena characteristic of a mild infection.

The time of development of the metastatic orchitis as reckoned from the day of inoculation is given in Table II. The mean value for the control groups was 51.0 days as compared with 53.7 days for the vaccine-immune animals, a difference of 2.7 days. The differences in the first and third experiments were 4.7 and 9.0 days respectively, but in the second experiment, the order of development was reversed, the metastatic orchitis of the vaccine-immune group antedating that of the controls by 5.0 days. Reference to the curves of Text-fig. 1 shows that in the first and third experiment, the metastatic orchitis of the controls uniformly preceded that of the vaccine-immune animals, while in the second experiment, the curve for the immunes precedes that for the controls except at one point.

The result in the second experiment is in keeping with the character of the earlier phenomena in consequence of which a restraining effect upon the subsequent phenomenon of the metastatic orchitis failed to develop. There are at least two reasons for this behavior of the

vaccine-immune animals of this second series. One must first take into account the known variability of reactive phenomena characteristic of a mild infection, the type which prevailed in this experiment. In the second place, the period of vaccine immunity (84 days) was much longer than in the other experiments (20 and 30 days). It is quite likely that if a state of initial hypersensitiveness leading eventually to a more effectual reaction, existed in the first experiment as was suggested by the observations in connection with the primary orchitis and critical edema, it might not continue over a prolonged period.[2] The curves representing the incubation period (Text-fig. 1, Experiment II) show only a slight difference in favor of an earlier development of the primary orchitis on the part of the vaccine-immune group, while the low incidence of edema makes a comparison of this phenomenon unsatisfactory, although the curve for the vaccine-immunes does precede that for the controls. There is no clearly defined group difference in the progress of the infection up to this point as far as the time relations and the incidence of reactive phenomena are concerned. But with the development of the metastatic orchitis, the vaccine-immune group preceded the controls, and the difference was of the same order and degree as that observed with the earlier reactions of the first experiment and resulted in a similar restraining or inhibiting effect upon later phenomena, in this case the development of generalized lesions (Text-fig. 1). In the first experiment, the shorter incubation period of the primary lesion and the earlier development and high incidence of the critical edema in the immune animals as compared with the controls was followed by a definite delay in the development of the next phenomena, namely, the metastatic orchitis and the generalized lesions. In the third experiment, group differences in the time relations of the primary orchitis and critical edema are not so clear-cut as in the first experi-

[2] It should also be noted that the rabbits of the second vaccine immune groups had been living under laboratory conditions approximately 3 months longer than the controls. This factor of caging and of age as well, undoubtedly had some influence upon the character of the reaction, but it is difficult of estimation, for while its effect is usually seen in the direction of increased efficiency, or in what may be termed a lower initial reactive state, such effects are not invariable or constant.

ment, but they become well marked with the development of the metastatic orchitis and again with the time of appearance of the first generalized lesions, as shown by the curves of Text-fig. 1.

The development of a metastatic orchitis in the doubly inoculated animals occurred in 50.8 and 58.2 days after inoculation (Table II). As compared with the controls, it was delayed 4.6 days in the first experiment, but in the second it preceded the control value by 3.6 days. The small magnitude of these values is of especial interest in that a greater delay in the development of a metastatic orchitis might be expected in view of the profound disturbance of the primary orchitis caused by the early vaccinal reaction. In the first experiment, the lesion of the uninoculated testicle developed 16.6 days earlier than that of the inoculated testicle, while in the second, the results were reversed, the primary orchitis developing 13.7 days before the metastatic lesion. But it should be noted that despite these differences, the development of the metastatic orchitis in both experiments occurred in comparable periods of time and curiously enough there was less difference between them (8.4 days) than between the corresponding control values (15.3 days).

Generalized Lesions.—The phenomenon of generalized lesions is the most significant single basis for estimating the general character of the syphilitic reaction. The occurrence or non-occurrence of generalized manifestations, the time of their appearance, their number, nature and persistence are among the features of this phase of the infection which may be used in evaluating its character.

The mean incidence of generalized lesions was practically the same in the control and vaccine-immune groups as shown in Table I and there were no significant differences in individual experiments except in the first. Here the disease was very severe and all controls developed secondary manifestations, but there was one vaccine-immune animal in which none was detected. In the doubly inoculated rabbits, on the other hand, there was a low incidence of generalized lesions, the mean value of 30.0 per cent contrasting with 86.7 per cent for the controls (Table I).

In regard to the distribution of generalized lesions, the mean values given in Table I show definite group differences. The following

analysis gives the extent of these differences in percentage terms of the control values:

Group	Actual rate	Relative rate
	per cent of control value	*per cent of control value*
Controls	8.6	7.4
Vaccine-immunes	6.3 or −26.72	5.6 or −24.32
V.V. (I.T.)	7.7 or −10.46	2.3 or −68.92

As has been stated in the section on Material and Methods, the relative rate of focal distribution which is estimated upon the total number of animals in the group, gives a more comprehensive idea than the actual rate of the extent of the lesions irrespective of the number of animals affected. This point is illustrated by the actual and relative rates for the doubly inoculated animals as shown in the above table. The magnitude of the actual rate is disproportionately large, due to 2 cases of moderately severe syphilis and the relative rate is a fairer expression of the group as a whole. From the figures given above, it is clear that as far as the distribution of generalized lesions is concerned, the infection of the doubly inoculated animals was much less pronounced than that of the controls, and while in the vaccine-immunes it was not as mild as in the doubly inoculated rabbits, still it was definitely less severe than in the controls.

In regard to the initiation of this phase of the reaction as determined clinically, the mean values as given in Table II show that generalized lesions appeared first among the controls and last among the doubly inoculated animals. The difference in time as compared with the control value was 5.6 and 17.1 days for the vaccine-immune and doubly inoculated rabbits, respectively, and as far as individual experiments were concerned, the results for both groups were consistent. In the first and third experiments in which the disease was severe, the delay in the appearance of generalized lesions in the vaccine-immunes as compared with the controls amounted to 9.3 and 7.7 days respectively. The delay in the second experiment was only 1.3 days, a negligible difference from the standpoint of magnitude, but of some importance in that it conforms to the results of the other experiments. That a greater difference did not occur is probably due

to the mild character of the disease. In the case of the doubly inoculated animals of the first experiment, generalized lesions first appeared 10.8 days after the controls while in the second series, their appearance was delayed 27.0 days beyond the control group. The difference in the character of the disease probably explains the difference in the magnitude of these results, that is to say, the effect induced by the double inoculation in the direction of delayed reaction was to some extent offset in the first experiment by the severity of the infection.

With regard to the last generalized lesions to appear, as shown in Table II and Text-fig. 1, there was little difference between the vaccine-immune and control animals. But with the doubly inoculated rabbits, the development of the few generalized lesions which did appear was so delayed that the mean value for the last lesion is considerably larger than the control figure, that is, 90.7 and 75.1 days respectively.

Further differences in the reaction of the vaccine-immune groups as compared with the controls are brought out in the matter of the time relationships existing between the initiation of the phenomenon of generalized lesions and those which preceded it as shown in the following table:

From the Primary Orchitis to the First Generalized Lesions.

Group	Experiment I	Experiment II	Experiment III	Mean values
	days	*days*	*days*	*days*
Controls	35.2	39.1	35.4	35.8
Vaccine-immunes	48.3	42.5	44.3	44.2
From the Critical Edema to the First Generalized Lesions.				
Controls	17.0	21.6	13.7	16.9
Vaccine-immunes	28.7	30.8	26.0	27.7
From the Metastatic Orchitis to the First Generalized Lesions.				
Controls	1.5	0.2	2.3	0.6
Vaccine-immunes	7.1	6.5	1.0	3.5

Considering only the mean values given above, the reaction intervals of the vaccine-immune animals exceeded those of the controls to the following extent:

	per cent
From the primary orchitis to the first generalized lesion.....	23.5
From the critical edema to the first generalized lesion.....	63.9
From the metastatic orchitis to the first generalized lesion.....	48.3

It has already been pointed out that a critical edema is a variable phenomenon and this fact together with the difference in the number of animals composing the 2 groups, contributes to the magnitude of the value resulting from the comparison of the critical edema and generalized lesion interval. But the above analyses show very clearly that the reaction of the vaccine-immune rabbits in the case of the generalized manifestations of disease was not as prompt as that of the control animals. Similar comparisons with the doubly inoculated rabbits are not applicable because of the irregularities already noted in connection with the early phenomena of the infection.

A significant aspect of the reaction to syphilis is the duration of the period in which generalized manifestations develop. In the following table the controls and the vaccine-immune animals are compared from this standpoint, using the values in Table II for the time of appearance of the first and last generalized lesions, and it is at once apparent that this phase of the reaction was considerably shortened in the case of the vaccine-immune groups.

Duration of Active Period of Generalized Lesions.

Experiment	Controls	Vaccine-immunes	Difference in time	Per cent of control values
	days	*days*	*days*	*per cent*
I	30.8	21.7	−9.3	or 30.2
II	18.4	10.0	−8.0	or 43.5
III	20.0	14.8	−5.2	or 26.0
Mean values	23.5	15.5	−8.5	or 36.2

In regard to the doubly inoculated animals, the active period of generalized manifestations in the first experiment was 27.5 days as compared with 30.8 days for the controls (Table II). It will be remembered, however, that in this experiment the incidence of generalized lesions was 40.0 and 100.0 per cent for the doubly inoculated and control groups respectively (Table I) and although this period of eruptive activity was but slightly shortened in the case of the

doubly inoculated groups, the compared value represents only 2 animals. In the second experiment only 1 doubly inoculated rabbit (20.0 per cent) developed 2 generalized lesions separated by an interval of 11 days as compared with an incidence of 70.0 per cent among the controls and a period of active development of 18.4 days (Tables I, II and III). It would appear, therefore, that there was a definite tendency on the part of the doubly inoculated rabbits toward a shortening of the active period of generalized manifestations as was the case with the vaccine-immune rabbits.

The mean time of appearance of *all* generalized lesions together with their time distribution may also be used as a basis of comparing the syphilitic reaction. The majority of lesions in the vaccine-immune and doubly inoculated animals tended to develop later than in the controls, as indicated by the mean values given in Table II, that is, 66.6 and 71.4 days as compared with the control figure of 61.0 days, but the difference is more strikingly brought out in the distribution curves of Text-fig. 2. Since the relative focal distribution rates have been employed in making these curves, their height gives an idea of disease severity. The delay in the initiation of secondary lesions, the generally lower level of the curves and the tendency for the majority of lesions to develop late in the course of the disease are uniform features of the reaction in the vaccine-immunes and doubly inoculated animals not observed in the controls.

Resolution and Healing of Lesions.—The last phase of the reaction to be considered is the state of the lesions at the conclusion of the experiment, that is, 3 months after inoculation. By this time, in a group of 5 or 10 rabbits and under ordinary conditions of intratesticular inoculation with a virulent strain of *T. pallidum*, a certain number of animals still show active lesions; in others, the lesions are regressing, while in the remainder, the manifestations of the disease are healed. A comparison of experimental groups from this standpoint furnishes the basis, therefore, for estimating the character of the syphilitic reaction in its capacity toward the accomplishment of a latent state. The following table summarizes the observations on the state of the lesions in the various groups of the present experiments 3 months after inoculation.

Final State of the Lesions.

Experiment	Healed		Not healed		Active	
	No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
I C	1	10.0	5	50.0	4	40.0
V.V. Im.	1	25.0	2	50.0	1	25.0
V.V. (I.T.)	1	20.0	2	40.0	2	40.0
II C	0		6	60.0	4	40.0
V.V. Im.	3	60.0	2	40.0	0	
V.V. (I.T.)	3	60.0	2	40.0	0	
III C	0		10	100.0	0	
V.V. Im.	2	20.0	8	80.0	0	
Mean values						
C	1	3.3	21	70.0	8	26.7
V.V Im.	6	31.6	12	63.2	1	5.3
V.V. (I.T.)	4	40.0	4	40.0	2	20.0

This analysis shows that in the late stage of the disease the activity of the syphilitic process as measured by the state of the lesions was much less pronounced in the vaccine-immunes than in the controls as shown by contrasting the percentage of animals in which the lesions either were healed or were still active. In the case of the doubly inoculated rabbits, a similar result obtained as regards the proportion of animals with healed lesions but not with respect to those which still showed active manifestations. This variant result was due to 2 doubly inoculated rabbits in the first experiment in which the disease was of average severity as far as the focal distribution of generalized lesions was concerned, although their appearance was delayed beyond those of the controls (Tables II and III; Text-fig. 1), so that active manifestations were present in these animals 3 months after inoculation. And it should be emphasized that these 2 rabbits were the only ones in either the first or second groups of doubly inoculated animals which developed a disease of even average severity; in all others it was extremely mild. If the mean values of the above table are combined on the basis of the presence or absence of syphilitic lesions, irrespective of degree of activity, a distinct difference between the vaccine-immunes and doubly inoculated animals on the one hand, and the controls on the other, is again brought out.

Group	Lesions healed	Lesions present
	per cent	*per cent*
Controls	3.3	96.7
Vaccine-immunes	31.6	68.5
V.V (I.T.)	40.0	60.0

From the foregoing discussion of the reaction to syphilis in rabbits immunized to vaccine virus and in rabbits inoculated intratesticularly with *T. pallidum* and vaccine virus, it is clear that the reaction of both sets of animals differed from that of the controls. As far as the vaccine-immune rabbits were concerned, the time relations of the early phenomena, that is, the primary lesion and the critical edema, were shorter than in the control groups, suggesting an initial condition of hypersensitiveness. But the subsequent manifestations of a metastatic orchitis and of generalized lesions developed later than in the controls, and in addition, the distribution rates of generalized lesions were lower, the period of activity of this phase of the infection was much shorter, and in a higher proportion of animals, all lesions had become healed 3 months after inoculation. From the standpoint of the entire disease picture, therefore, it is evident that the effectiveness of the host's reaction was increased by the presence of vaccine immunity at the time of inoculation with syphilis.

With respect to the animals inoculated simultaneously in the testicle with *T. pallidum* and vaccine virus, the results on the whole conform to those obtained in the vaccine-immune rabbits as far as the later phases of the syphilitic reaction were concerned, including the important one of generalized lesions. The results connected with the early syphilitic phenomena were of an entirely different order from those which obtained in the vaccine-immunes and in the controls due, presumably, to the local effects of the vaccinal reaction. The modification of the syphilitic reaction in the doubly inoculated animals in the direction of increased effectiveness has been interpreted as due, in part at least, to the effect of a vaccinal immunity. It is known that vaccine immunity in rabbits is well developed 2 to 3 weeks after inoculation of the virus and continues for an indefinite time. The influence of such an immune state upon the syphilitic reaction would, therefore, be operative during the period of development of syphilitic

phenomena, and the present experiments show that the modification of the syphilitic reaction was of the same general order as that observed in rabbits immune to vaccine virus at the time of *pallidum* inoculation.

Mention was made at the beginning of this paper that the results obtained with a certain strain of *T. pallidum* were entirely unexpected in that the strain is reported to be capable of producing a severe disease and that which developed in our rabbits was extremely mild. It was found that the original rabbit from which we obtained the strain was also infected with vaccine virus and furthermore, that the virus was being transferred with the syphilitic inocula. In the light of the experiments reported in this paper, the reason for these unexpected results is now clear, namely, a more effective reaction to the syphilitic infection associated with the development of vaccinal immunity. To what extent the results were also influenced by the local reaction from the contaminating vaccine virus is difficult to say, but it is certain that the vaccinal reactions were not as marked in the experiments referred to above as those in the experiments described in the present paper.

The results reported in this paper are entirely different from those obtained in other experiments in which vaccine virus and *T. pallidum* were also inoculated simultaneously but in different sites, that is, vaccine virus intracutaneously on the side of the body and *T. pallidum* intratesticularly (1). Under these conditions of inoculation, the effectiveness of the syphilitic reaction was greatly decreased, resulting in a severe infection, and this despite the development of an intense vaccinal reaction and a subsequent vaccinal immunity. It is evident, therefore, that the manner in which two concomitant experimental infections are induced is an important factor in determining the nature and capacity of the host's reaction to one of them.

SUMMARY.

Experiments are reported in which it was shown, first, that the effectiveness of the reaction to experimental syphilis was increased in rabbits immune to vaccine virus, the ensuing disease being less severe than in control animals.

It was further shown that a comparable modification of the syphilitic reaction occurred in rabbits inoculated intratesticularly with a mixture of *Treponema pallidum* and vaccine virus, and it was suggested that this result was due to the influence of a vaccinal immunity and to the local effect of the vaccinal reaction.

It was pointed out that the manner in which two concomitant experimental infections are induced is of significance in determining the character of the host's reaction.

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THE INCUBATION PERIOD OF YELLOW FEVER IN THE MOSQUITO.

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During the yellow fever epidemic at Orwood and Taylor, Mississippi, in 1898, Carter (1, 2) noted that an interval of from 2 to 3 weeks elapsed between the first infecting cases and the secondary cases arising therefrom. This observation antedates, of course, the demonstrations of mosquito transmission of the disease. In 1900 Reed, Carroll, Agramonte and Lazear (3) in establishing the fact of mosquito transmission discovered that a period of about 12 days after biting was required before the mosquito was capable of inducing the infection in normal individuals. They showed, for example, in one experiment, that several mosquitoes which had fed on yellow fever patients, were non-infective on the 4th and 7th days and infective to the same individual on the 17th day following the original feeding. Guiteras (4) confirmed this finding. Mosquitoes having fed on a yellow fever patient were non-infective to a susceptible person on the 5th and infective on the 20th day after the feeding. Marchoux, Salimbeni and Simond (5) who made numerous such tests concluded that at least 12 days must elapse between the feeding of mosquitoes (*Aedes ægypti*) on yellow fever patients and their ability to transmit the infection to fresh susceptible persons. This period between the initial infecting feeding and the time at which the mosquitoes are capable of inducing infection explains and corresponds to what has been called by Carter "extrinsic incubation."

Now that it has been shown (6) that the virus of yellow fever is transmissible to *Macacus rhesus*, in which species of monkey an experimental disease is produced corresponding to yellow fever in man, it becomes possible to determine more precisely the exact period of this extrinsic incubation. Earlier experiments (6) had shown that whereas

the virus is readily filtrable when in the circulating blood, the filtrates prepared from mosquitoes which had fed on infected animals were ineffective on inoculation. The experiments to follow were designed for the purpose of elucidating the different states in which the virus is supposed to exist in the mosquito during extrinsic incubation and in man and monkey during actual infection. A uniform number of mosquitoes were made to bite normal *Macacus rhesus* monkeys at various intervals after the infectious feed, after which they were ground up and injected into susceptible monkeys.

The temperature in the storage cage in which the mosquitoes were kept during these experiments was registered with a recording thermometer and was found to vary daily between 74° and 90°F.; the coolest time was between 6 and 8 o'clock in the morning and the warmest between 3 and 5 in the afternoon.

Experiment 1.—On January 28, 1928, a normal *rhesus*, No. 50–6, was exposed to Lot 27 of infected *A. ægypti*, eighteen of which fed upon this animal. On February 3 the monkey showed a temperature of 105.8°F., and on that date Lot 35, consisting of 95 normal females, *Aedes ægypti*, were made to feed on the animal.

At intervals of 48 hours, five mosquitoes of this lot were transferred to a suitable cage and a normal monkey exposed until each of these five insects had fed. The mosquitoes were then caught, killed with tobacco smoke, ground up in a mortar with 3.0 cc. of salt solution and the emulsion injected subcutaneously into another normal *rhesus* monkey. This procedure was carried out 2, 4, 6, 8, 10 and 12 days after the original feeding of the mosquitoes on *Rhesus* 50–6. The mosquitoes of this lot died very rapidly so that on February 15, *i.e.*, on the 12th day after the original feeding, only three remained alive, which were used instead of five.

As seen from Table I, the results of this experiment were irregular. None of the mosquitoes which fed on normal monkeys 2, 4, 6, 8 and 10 days after the original infectious feed induced disease, while those tested on the 12th day produced infection. On the other hand, 2, 4 and 8 day mosquitoes, which failed to infect by biting, proved infectious when used for injection into monkeys. 12 day mosquitoes produced infection by both methods of inoculation, while 6 and 10 day insects failed by biting and by injection. All monkeys failing to respond were shown to be susceptible by subsequent inoculation with virulent blood or by biting with known infected mosquitoes.

The length of life of a female *A. ægypti*, under laboratory conditions,

irrespective of whether the insect is infected with yellow fever virus or not, averaged in our experience about 2 months, and about 10 per cent of the insects usually lived over 3 months. Occasionally, however, some batches of mosquitoes became subject to epidemics in which all insects that happened to be in one cage died within a few days, irrespective of their age, food, surrounding temperature, humidity, etc.

TABLE I.

Experiment 1. Mosquitoes, Lot 35, Infected February 3, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 5	2	51-1	5 mosquitoes fed	No reaction
" 5	2	51-2	Same mosquitoes macerated and injected	Feb. 17. Died. Postmortem findings typical
" 7	4	51-3	5 mosquitoes fed	No reaction
" 7	4	51-4	Same mosquitoes macerated and injected	Feb. 13. Died. Postmortem findings typical
" 9	6	51-5	5 mosquitoes fed	No reaction
" 9	6	51-6	Same mosquitoes macerated and injected	No reaction
" 11	8	51-7	5 mosquitoes fed	No reaction
" 11	8	51-8	Same mosquitoes macerated and injected	Feb. 17. Died. Postmortem findings typical
" 13	10	51-9	5 mosquitoes fed	No reaction
" 13	10	52-0	Same mosquitoes macerated and injected	No reaction
" 15	12	52-1	3 mosquitoes fed	Feb. 26. Died. Postmortem findings typical
" 15	12	52-2	Same mosquitoes macerated and injected	Feb. 24. Died. Postmortem findings typical

According to Howard, Dyar and Knab (7), these epidemics have been studied by numerous workers and have been attributed mostly to fungi and occasionally to bacteria which are supposed to invade the digestive tract and the body cavities. Having no other explanation, it seems to us that the high mortality in the mosquitoes of Lot 35 was due to one of these parasitic infections. If this was the case, it seems also possible that the yellow fever virus might in the 6 and 10 day mosquitoes have been destroyed by the invading organisms. This

hypothesis seems to be supported by the results obtained in subsequent experiments.

Experiment 2.—*Rhesus* 51–8, which in the above experiment on February 11 had been inoculated subcutaneously with the emulsion of five macerated mosquitoes, continued to show normal temperature until February 15, when the temperature suddenly rose to 105.3°F. The monkey was immediately exposed to a lot of

TABLE II.

Experiment 2. Mosquitoes, Lot 36, Infected February 15, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 18	3	54-5	10 mosquitoes fed	No reaction
" 18	3	54-6	Same mosquitoes macerated and injected	Feb. 27. Died. Postmortem findings typical
" 20	5	54-7	10 mosquitoes fed	No reaction
" 20	5	54-8	Same mosquitoes macerated and injected	Feb. 27. Died. Postmortem findings typical
" 22	7	54-9	10 mosquitoes fed	No reaction
" 22	7	55-0	Same mosquitoes macerated and injected	Mar. 1. Died. Postmortem findings typical
" 24	9	55-1	10 mosquitoes fed	Mar 2. Died. Postmortem findings typical
" 24	9	55-2	Same mosquitoes macerated and injected	Feb. 29. Died. Postmortem findings typical
" 26	11	55-3	10 mosquitoes fed	Mar. 2. Died. Postmortem findings typical
" 26	11	55-4	Same mosquitoes macerated and injected	Mar. 6. Died. Postmortem findings typical
" 28	13	55-5	10 mosquitoes fed	Mar. 5–9. Fever. Recovered
" 28	13	55-6	Same mosquitoes macerated and injected	Mar. 8. Died. Postmortem findings typical

normal *A. ægypti*, of which 130 became engorged. These were labelled "Lot 36" and were used in the next experiment.

Beginning on the 3rd day after feeding on *Rhesus* 51–8, and thereafter on every other day, ten mosquitoes were transferred to a suitable cage and fed on a normal monkey. After the monkey was removed, the engorged mosquitoes were caught, killed with tobacco smoke, ground up in a mortar with 3.0 cc. of salt solution and the emulsion injected subcutaneously into another normal monkey. This procedure was carried out on the 3rd, 5th, 7th, 9th, 11th and 13th days from the date when the mosquitoes had originally fed on the infected monkey. No mortality

occurred among the mosquitoes of this lot to interfere with the completion of the experiment.

The results are summarized in Table II. This experiment shows that the virus is definitely infectious during the entire period of the extrinsic incubation. Typical yellow fever was produced in monkeys by injecting the substance of the mosquitoes at any time after the insects had ingested the virus from an infected animal. Although then present in the mosquitoes in an infectious form, the virus was not, however, transmitted by bite until from the 9th day on. As seen from this table, all the animals which developed infection, either as a result of the injection of mosquito emulsion or through bite of the infected insects, died with the exception of one, *Rhesus* 55–5, which had been bitten by the 13 day mosquitoes. 5 days after the bite this monkey developed high temperature which continued for another 5 days. Final recovery ensued and upon subsequent inoculation with a large dose of virulent blood, the animal again ran a febrile period but did not succumb. The three monkeys which were bitten by 3, 5 and 7 day mosquitoes and did not respond, were found as susceptible as normal animals to later inoculation with virulent blood.

In order to confirm these findings, one more experiment was carried out, with the same technique and the same number of mosquitoes, but over the time intervals at which Experiment 1 was performed.

Experiment 3.—*Rhesus* 51–3, which in Experiment 1 had been bitten by 4 day mosquitoes without response, was on February 20 exposed to two infected mosquitoes, of which one only fed. On the afternoon of February 23, the animal's temperature was 106.0°F.; it was then exposed to a lot of normal *A. ægypti* and 102 of these became engorged. These, labelled "Lot 42," were used in the following experiment.

Beginning on the 4th day after feeding on *Rhesus* 51–3, and thereafter on every other day, ten of these mosquitoes were placed in a suitable cage and made to feed on a normal monkey. After the monkey was removed, the engorged mosquitoes were caught, killed with tobacco smoke and ground up in a mortar with 3.0 cc. of salt solution. The emulsion was injected subcutaneously into another normal monkey.

The results are given in Table III. All monkeys which had been inoculated with macerated mosquito emulsions at various intervals after the insects had their infectious feed, died of typical yellow fever infection. Those, on the other hand, which had been bitten by the

same mosquitoes at the same intervals, gave no response with the exception of one monkey, No. 56–5, on which 12 day mosquitoes were allowed to feed, and which died of typical yellow fever. Monkeys 55–7, 55–9 and 56–1 were later either inoculated with virulent blood or exposed to mosquitoes which were known to be infected and died of yellow fever, thus proving their susceptibility. However, Monkey 56–3, which was bitten by 10 day mosquitoes and showed no reaction,

TABLE III.

Experiment 3. Mosquitoes, Lot 42, Infected February 23, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 27	4	55-7	10 mosquitoes fed	No reaction
" 27	4	55-8	Same mosquitoes macerated and injected	Mar. 10. Died. Postmortem findings typical
" 29	6	55-9	10 mosquitoes fed	No reaction
" 29	6	56-0	Same mosquitoes macerated and injected	Mar. 8. Died. Postmortem findings typical
Mar. 2	8	56-1	10 mosquitoes fed	No reaction
" 2	8	56-2	Same mosquitoes macerated and injected	Mar. 11. Died. Postmortem findings typical
" 4	10	56-3	10 mosquitoes fed	No reaction; later proved immune
" 4	10	56-4	Same mosquitoes macerated and injected	Mar 16. Died. Postmortem findings typical
" 6	12	56-5	10 mosquitoes fed	Mar. 16. Died. Postmortem findings typical
" 6	12	56-6	Same mosquitoes macerated and injected	Mar. 12. Died. Postmortem findings typical

proved refractory when given 2 weeks later a large dose of virulent blood. It is impossible to determine whether the last animal was naturally immune or had acquired immunity as the result of the mosquito bite.

SUMMARY.

1. The yellow fever virus was found in infectious form in *Aedes ægypti* throughout the entire period of the extrinsic incubation, as demonstrated by the injection of the bodies of mosquitoes into normal

rhesus monkeys at daily intervals after the insects had fed on an infected animal.

2. The virus was transmitted through the bite of the mosquitoes, in one experiment on and after the 9th day, and in two experiments on the 12th day after the initial infecting feed.

3. The pathologic changes produced by the injection of the infected mosquitoes into normal monkeys during the extrinsic incubation were in every respect those of typical experimental yellow fever.

4. The monkeys withstand easily the subcutaneous injection of the mosquito emulsion. No acute inflammatory reaction was observed at the site of injection in any of the seventeen animals inoculated with this material in these three experiments.

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